PATHOPHYSIOLOGICAL CORRELATIONS IN MAEDI - A CHRONIC LYMPHOID INTERSTITIAL PNEUMONIA OF SHEEP

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Declaration

I declare that the contents of this thesis are my own work and that they have not been presented to any University other than the University of Edinburgh

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ABSTRACT

Maedi is a chronic lymphoid interstitial pneumonia (LIP) of sheep caused by the lentivirus, maedi-visna virus (MVV). Following a long prepatent phase maedi progresses to clinical disease characterised by exercise intolerance and dyspnoea. Although maedi is well characterised at a pathological level, physiological studies of the disease have not been undertaken. As a consequence, the nature of the functional abnormality that underlies the clinical disease is unknown, whether there exists measurable functional deficit in the preclinical phase of the disease is unknown and the relationship between lung pathology and functional deficit is unknown. These questions are fundamental to understanding the way in which pathological events ultimately conspire to bring about organ dysfunction and clinical disease. Further, knowledge of the way in which pathology relates to measurable lung dysfunction offers a potential means of assessing the progress and prognosis of this disease. This thesis describes an investigation into the pathophysiological mechanisms responsible for inducing lung functional deficits in maedi.

As a prelude to establishing the nature of the functional deficit in maedi, repeated measurements of static lung compliance (Cst), lung distensibility (K), effective alveolar volume ($V_{A,eff}$) and transfer factor for carbon monoxide ($T_{L,CO,'sb'}$) were made in anaesthetized control sheep, seronegative for MVV, over a period of 5 months. This study furnished regression equations and prediction intervals for lung function indices in normal sheep using bodyweight as the independent variable. By comparison with predicted normal values sheep naturally infected with MVV had reduced lung volumes and gas diffusing capabilities and increased lung elastic recoil.

A pathophysiological study was instigated to identify structural correlates of lung functional deficits. Preliminary investigation involved the quantitative morphometric characterisation of the normal sheep lung. Data from this study indicated that the ratio of fixed to physiological lung volume ranged from 0.49 to 0.59 and that this ratio was positively correlated with the time between euthanasia and inflation fixation of the lungs. Values for tissue volume fraction within the lung parenchyma (V_{vt}) ranged from 0.18 to 0.25 and values for alveolar surface density (S_{vt}) ranged from 592 to 716 cm^2/cm^3. Pathophysiological correlations in MVV-infected sheep indicated that lung volume and transfer factor measurements were more sensitive indices of pathology than measurements of Cst or K. Transfer factor was reduced even in sheep with minimal histopathology suggesting this index as a sensitive means of assessing this condition. The density of surface forces could not account for variation in K seen *in vivo*, however tissue factors such as the quantity and functional tone of contractile tissue in the parenchyma, airways or blood vessels may contribute. Given that parenchymal smooth muscle hyperplasia is a pathological feature of maedi, it was hypothesized that this tissue element is responsible for the observed reduction in K.

In order to further investigate this relationship, the distribution and morphometric quantitation of α-smooth muscle actin (ASMA) in lung parenchyma from normal and MVV-infected

sheep was determined and related to *in vivo* functional measurements. The volume density of ASMA (V_V'ASMA') was negatively correlated with K and Cst, however partial correlation coefficients indicated that V_V'ASMA' and V_{VT} were strongly interdependent thus complicating interpretation of the link between V_V'ASMA' and K. In order to separate the influence of dynamic and passive tissue elements, histamine and clenbuterol were administered to normal and MVV-infected sheep in an attempt to cause relaxation and contraction of parenchymal contractile tissue. The functional response of the cardiopulmonary system to intravenous infusion of these agents was measured and correlated with V_V'ASMA'. K and Cst were significantly increased following clenbuterol injection, however only the increase in K was correlated with the quantity of V_V'ASMA', and this correlation was negative. These results could be explained if the site of action of clenbuterol was not the contractile tissue at the level of the alveolar ducts, but rather that which surrounds conducting airways.The dose of histamine required to lower dynamic compliance to 65% of baseline values was negatively correlated with V_V'ASMA' and the attendant percentage change in K was positively correlated with V_V'ASMA'. These findings support the contention that parenchymal contractile tissue is of functional relevance and capable of regulating overall lung elastic properties.

Maedi is a naturally occurring disease in sheep in which the aetiologic agent and target cell is known and in which the pathology is well characterised. As such it has potential as a model for LIP associated with human immunodeficiency virus infection in children and adults. The present study has both served to establish the functional characteristics of this disease and indicate structural correlates of observed functional deficits. Moreover, evidence is presented to suggest that the observed reduction in lung distensibility in maedi is a consequence of increased tissue forces associated with the parenchymal smooth muscle hyperplasia that is a feature of this disease.

INDEX

Abbreviation	Initial text reference	
A	3.2.5	Difference between Vmax and the intercept on the volume axis
ABC	8.2.3	Avidin-biotin complex
AEC	8.2.3	3-amino-9-ethylcarbazole
AGID	1.4	Agar gel immunodiffusion
AIDS	4.1	Acquired immunodeficiency syndrome
ASA_p	5.2.6	Total airspace surface area of lung parenchyma
ASMA	8.2.3	α-smooth muscle actin
BALF	1.9.2	Bronchoalveolar lavage fluid
BEvt	9.4	Base excess
BP	9.2.4.1	Blood pressure
C_{dyn}	9.2.4.2	Dynamic compliance
COE	2.2.3	Carbon monoxide concentration in the expired sample
COI	2.2.3	Carbon monoxide concentration in the inspired sample
Cst	2.2.3	Static compliance
DAB	8.2.3	Diaminobenzidine tetrahydrochloride
$ED_{65}C_{dyn}$	9.2.6.3	Dose rate required to lower C_{dyn} to 65% of baseline values
$ED_{200}R_L$	9.2.6.3	Dose rate required to increase R_L twofold
ELISA	1.4	Enzyme-linked immunosorbent assay
E_{oes}	9.2.4.2	Oesophageal elastance
FRC	2.2.3	Functional residual capacity
HeE	2.2.3	Helium concentration in the expired sample
HeI	2.2.3	Helium concentration in the inspired sample
HIV	1.3	Human immunodeficiency virus
HR	9.2.4.2	Heart rate

ILD	1.9.2	Interstitial lung disease
K	3.2.5	Index of distensibility
LIP	4.1	Lymphoid interstitial pneumonia
LIP-HIV	1.9.2	LIP associated with HIV infection
LLW	5.2.2	Left lung weight
L_m	App 7.12	Mean linear intercept
LTR	1.3	Long terminal repeat
MVV	4.1	Maedi-visna virus
O_2sat	9.4	Oxygen saturation
ORF	1.3	Open reading frame
P	3.2.5	Static recoil pressure
$PaCO_2$	9.4	Arterial carbon dioxide tension
Pao	9.2.4.3	Airway opening pressure
PaO_2	9.4	Arterial oxygen tension
PBS	8.2.3	Phosphate-buffered saline
Ptp	2.2.2	Transpulmonary pressure
R_{int}	9.2.4.3	Airway resistance by the interrupter method
R_L	9.2.4.2	Total pulmonary resistance
sE_{oes}	9.2.4.2	Specific oesophageal elastance
s_t/v_t	5.4	Surface to volume ratio for tissue
S_{vt}	5.2.6	Tissue surface density
tCO_2	9.4	Total carbon dioxide content in blood
TGF-β	8.5	Transforming growth factor β
$T_{L,CO}$	2.2.3	Transfer factor for carbon monoxide
$T_{L,CO,'sb'}$	2.2.3	Single-breath transfer factor for carbon monoxide

$T_{L/VA}$	2.2.3	Transfer factor per litre of effective alveolar volume
TLC	2.2.3	Total lung capacity
TLV_F	5.2.4	Total fixed lung volume
TLW	5.2.2	Total lung weight
TV_p	5.2.6	Volume of lung parenchyma
$TV_{p,a}$	5.2.6	Total parenchymal airspace volume
$TV_{p,t}$	5.2.6	Total parenchymal tissue volume
V	9.2.4.1	Respiratory flow rate
$V_{A,eff}$	2.2.3	Effective alveolar lung volume
VD,an	2.2.3	Anatomic dead space volume
VD,eq	2.2.3	Instrumental dead space volume
VI	9.2.3.2	Virtual instrument
Vmax	3.2.5	Volume asymptote
Vsyr	2.2.3	Syringe volume used to inflate the lungs
$V_{v'ASMA'}$	8.2.3	Volume density of ASMA
V_{va}	5.2.6	Volume density of air
V_{vp}	5.2.4	Volume fraction of lung parenchyma
V_{vt}	5.2.6	Volume density of tissue

CHAPTER 1

MAEDI

1.1 Introduction

Maedi is a descriptive Icelandic term meaning 'dyspnoea' that is used to describe the respiratory component of the disease called maedi-visna which affects sheep in most parts of the world and is caused by infection with a lentivirus. The other components of this disease concern an encephalitis, arthritis and mastitis. Pathologically, maedi is classified as a chronic progressive lymphoid interstitial pneumonia. Progression of this disease invariably leads to physical incapacitation and death, therapeutic intervention being of negligible value.

1.2 History

1.2.1 Worldwide

The first description in the literature of a chronic lung disease with the characteristics of maedi dates from 1915 in South Africa (Mitchell, 1915). Thereafter, the disease was described in various parts of the world and often named with local descriptive terms or according to location e.g. 'Zwoegers' in Holland, Montana sheep disease in the U.S.A. (Marsh, 1923), Graaff-Reinet disease in South Africa (de Kock, 1929) and 'la Bouhite' in France (Lucam, 1942).

The greatest single epidemic of the disease occurred in Iceland following the importation of infected Karakul sheep from Germany in 1933. A successful eradication programme involving slaughter of infected sheep and replacement with sheep from non-affected districts was instituted and implemented over the next three decades. Within this time over 105,000 sheep succumbed to maedi and 650,000 sheep were slaughtered as part of the eradication programme (Pálsson, 1976). The Icelandic experience provided the basis for comprehensive study of the epidemiology and natural history of this disease and the concept of slow infections was borne of this experience (Sigurdsson, 1954). The essential features of slow infections are: (a) a long and unpredictable incubation period of months to years, during which time the agent produces clinically inapparent but progressive pathologic damage; and (b) a protracted course, once clinical signs have appeared, generally ending in serious disease or death (Sigurdsson, 1954).

Currently maedi-visna virus is present in most countries in Europe. It is also present in countries in the Middle East, several countries in Africa and Asia and also North and South America (Pálsson, 1976; Houwers, 1990).

1.2.2 History of maedi in the United kingdom

The first serological evidence of maedi-visna infection in a sheep flock in the U.K. was in 1978 (Dawson *et al.*, 1979). Although between 1979 and 1983, 170 infected flocks were identified in the U.K. (Pritchard *et al.*, 1984), only one clinical description of maedi appeared in the literature (Jones *et al.* 1982). Over the last decade, attempts at establishing the seroprevalence of maedi-visna virus infection have been limited, however Pritchard & Dawson, (1987) in a study of 23 commercial breeding flocks in East Anglia, identified 4 (17%) with one or more seropositive sheep. Investigative field-based research of maedi has frequently identified intercurrent lung pathologies and infections which have complicated clinical, epidemiological and pathological interpretation (Markson *et al.*, 1983; Pritchard *et al.*, 1984; Pritchard & Dawson, 1987; Pritchard & Done, 1990). However subsequent research has demonstrated that primary, uncomplicated maedi-visna virus infection can occur in flocks and that infection is associated with clinical and pathological evidence of disease affecting the respiratory, nervous, musculoskeletal and mammary systems (Watt *et al.*, 1992).

1.3 Aetiology

Maedi-visna virus (MVV) is the prototype of slow infections characteristic of the *Lentivirinae* subfamily of the family *Retroviridae*. Other viruses within the group include caprine arthritis encephalitis virus (CAEV) and human immunodeficiency virus and are listed in table 1.1 below.

Table 1.1

Virus	Abbreviation	Reference
Maedi-visna virus	MVV	Sigurdsson *et al.*, 1960
Caprine arthritis-encephalitis virus	CAEV	Crawford *et al.*, 1980
Feline immunodeficiency virus	FIV	Pedersen *et al.*, 1987
Simian immunodeficiency virus	SIV	Daniel *et al.*, 1985
Bovine immunodeficiency virus	BIV	Van Der Maaten *et al.*, 197
Equine infectious anaemia virus	EIAV	Kobayashi, 1961
Human immunodeficiency virus	HIV	Barré-Sinoussi *et al.*, 1983

Appearance of the virion at the electron microscopic level reveals a circular outer envelope covered in spikes surrounding an electron dense nucleoid core. The core of the virus contains two identical single-stranded RNA molecules of approximately 9-10 kilobases in length. All retroviruses have a similar genome arrangement in that they contain three major structural genes: *gag* coding for the core proteins of the virus, *pol* coding for reverse transcriptase and protease enzymes and *env* coding for the envelope glycoprotein. At either end of the genome are identical base sequences called long terminal repeats (LTRs) which are important for permitting and controlling transcription of the RNA to DNA, for the integration of the DNA into the host cell DNA and for the transcription of proviral DNA back to RNA. The presence of open reading frames (ORFs) found between the major structural genes distinguishes the lentiviruses from other retroviruses. These ORFs code for proteins which play a role in regulating the molecular events of virus replication.

Translation of the *gag* gene produces a protein of 55 kDa. This is a precursor protein which is cleaved into the three major core proteins p30, p16 and p14 (Vigne *et al.*, 1982). A 150 kDa precursor protein translated from *gag* and *pol* is split to yield the reverse transcriptase and *gag*-related proteins (Vigne *et al.*, 1982). Cleavage of the *env*-encoded precursor protein yields the outer membrane glycoprotein gp135 and transmembrane glycoprotein gp46. Products of the non-structural control genes include a transactivator of viral transcription (Tat, 10kDa) (Hess *et al.*, 1985) and a polypeptide necessary for the cytoplasmic expression of *env*-mRNA (Rev, 19kDa) (Tiley *et al.*, 1990). Other proteins encoded by the small ORFs include a 29kDa protein encoded by the *vif* gene, the function of which is unclear (Carey & Dalziel, 1993).

MVV replicates productively and lytically in tissue cultures in a matter of days (Thormar, 1961). In contrast, replication appears to be restricted *in vivo* leading to low virus titres (Brahic *et al.*, 1981). Studies of *in vitro* replication demonstrate that MVV enters the cell by fusion. The single-stranded viral RNA is reverse transcribed to form double-stranded DNA (dsDNA) of which the majority is in the form of linear molecules and a small fraction in a circular form (Clements *et al.*, 1979; Harris *et al.*, 1981; Haase *et al.*, 1982). Viral genes are transcribed from the dsDNA which also serves as the template for RNA that will be packaged within virions. The restriction that is observed *in vivo* appears to act at the level of transcription in that although many cells may contain considerable quantities of proviral DNA following experimental infection only a very small number of cells appear to express either major structural proteins (Haase *et al.*, 1977) or viral RNA (Brahic *et al.*, 1981).

Virus can be recovered from a variety of central nervous system and lymphoid tissues, however virus titres are minimal and often require the use of tissue explant techniques for isolation (Pétursson *et al.*, 1976). Evidence from virus isolation studies on peripheral blood suggests that only a very low proportion of mononuclear cells carry the virus (1 in 10^5 - 10^6 cells)(Pétursson *et al.*, 1976). The major target cells for infection are cells of the monocyte-macrophage lineage (Narayan *et al.*, 1982) and the level of viral replication is related to the differentiation of these cells (Gendelman et al, 1986). In natural disease, the total number of virus infected alveolar macrophages, as well as the ratio of infected to uninfected macrophages increases with the severity of pathology (Brodie *et al.*, 1992). Accumulated evidence thus suggests that the alveolar macrophage plays a crucial role in the pathogenesis of maedi.

1.4 Transmission and diagnosis

Although antenatal infection is thought to be of little relevance (Houwers *et al.*, 1989; Houwers 1990), lactogenic or ewe-to-lamb contact transmission is believed to be important (De Boer, 1979). Indeed the ewe-progeny relationship is particularly important in that progeny of seropositive ewes are much more likely to seroconvert than progeny of seronegative ewes (Houwers *et al.*, 1989). Conclusive evidence to support lactogenic or ewe-to-lamb contact transmission has not been reported.

The existence of horizontal transmission was conclusively demonstrated in Iceland where primary spread within flocks was initially very rapid and thereafter infection spread more slowly to uninfected flocks. Management practices i.e. housing during the winter months, communal grazings and autumn roundups of sheep from several different sources all contributed to the rapid horizontal spread of the disease within Iceland (Pálsson, 1976). Droplet infection via the respiratory route was considered the most likely method of transmission in the Icelandic epidemic (Pálsson, 1976). Other management factors such as selective breeding policies can have highly significant effects on the rate of horizontal transmission (Houwers, 1990). There is no evidence to support transmission via semen (Houwers, 1990).

Only a small number of cells in the body are infected following natural infection, therefore detection of viral particles, proteins or nucleic acids in blood or body fluids is not a viable method of detecting MVV-infected sheep (Hoff-Jorgensen, 1990). Detection of antibodies against MVV is the only method of diagnosis suitable for living animals. Currently used techniques centre around the agar-gel immunodiffusion (AGID) test and the enzyme-linked immunosorbent assay

(ELISA). The former test, although simple and reproducible, is time consuming and subjective, whereas the latter method can be automated and interpretation can be rendered objectively.

1.5 Pathology

Gross pathological features of maedi are apparent on opening the thorax. The affected lungs tend not to collapse, thus appearing enlarged and have a grey-brownish colouration. In advanced cases, rib impressions may be visible on the pleural surface. They are heavier than normal, the increase in weight occurring in the absence of oedema or consolidation. The cut surface is dry and the texture of the parenchyma is firm and rubbery. Close inspection of pleura and cut surface reveals small (1-2mm) grey-white foci. Caudal mediastinal and tracheobronchial lymph nodes become grossly enlarged. Pathological change seems to progress evenly throughout the lung parenchyma (Sigurdsson, 1954; Georgsson & Pálsson, 1971; Georgsson *et al.*, 1976).

Histopathology is characterized in the early stages by the development of lymphoid follicles either associated with blood vessels and airways or sometimes within the parenchyma apparently unrelated to such structures. With advancing pathology there is an increasing interstitial infiltrate of mononuclear cells, macrophages and lymphocytes. This infiltrate causes a progressive thickening of alveolar septae. Eventually the septae become thickened to the point that alveolar airspaces are obliterated. A frequent occurence is the hyperplasia of smooth muscle at the level of the alveolar ducts and at the tips of alveolar septae. Histologically identifiable smooth muscle may eventually appear within the thickened septae themselves, a site not normally associated with contractile tissue (Miller, 1921). Fibrosis is not a prominent feature of the histopathology although elastic fibres are fewer and fragmented (Sigurdsson, 1954; Georgsson & Pálsson, 1971; Georgsson *et al.*, 1976).

1.6 Clinical features

Because of the long incubation period that is characteristic of lentivirus infections, clinical signs of maedi do not commonly occur before 3 to 4 years of age (Pálsson, 1990) although occurrence in 2 year-old sheep has been reported (Sigurdsson, 1954; Watt *et al.*, 1992). The earliest clinical feature is usually exercise intolerance manifested as excessive dyspnoea when compared with unaffected sheep in the flock. There is obvious tachypnoea often with the neck extended, nostrils flared and abdominal type breathing. Open mouth breathing becomes apparent with disease progression and the level of exercise stress required to induce symptoms declines. Affected sheep are non-febrile, coughing is an inconsistent feature and auscultatory findings often only indicate an

increase in normal breathing noises, no adventitious noises being audible. Sheep usually are of bright demeanour despite clinical symptoms being apparent and only latterly will loss of body condition become obvious. Given sympathetic handling sheep may survive periods of months to years after the discasc has reached the clinical stage (Pálsson, 1990; Watt *et al.*, 1992).

1.7 Control

No vaccine is available to protect against MVV-infection. Control options currently available therefore depend on prevention and eradication programmes (Houwers, 1990). As outlined by Houwers (1990), the stratification of the sheep industry results in a flow of breeding product from the pedigree hill flocks through the upland production flocks to the lowland commercial flocks producing finished lambs for slaughter. The logical target for implementing voluntary or compulsory adoption of MVV-free status is amongst the producers of pedigree breeding and production sheep. Within the U.K., control at the national level is implemented through the Sheep and Goat Health Scheme which was launched in 1982 with the aim of providing a pool of sheep flocks and goat herds of recognised health status. To achieve accredited status a flock must undergo at least two clear blood tests separated by 180-360 days. Thereafter periodic testing of either all or a proportion of sheep over 18 months of age is implemented at initially yearly and then two-yearly intervals. Implementations are also made regarding positive test results, movement controls, isolation procedures, approved shows and markets, fencing and purchasing of animals. There are registration fees, yearly membership fees and sample fees which are paid by the flock owner. Currently, the AGID test is used to determine serological status.

At the flock level, control options include total replacement of the whole flock with MVV-free stock, repeated testing and culling of seropositives, artificial rearing of colostrum-deprived lambs and 'dilution' of infection through the purchase of MVV-free replacements (Houwers, 1990).

1.8 Economics

Areas of economic loss from flocks with MVV infection concern both direct and indirect effects (Houwers, 1990). In the former category are the losses associated from the loss of infected sheep that succumb to clinical disease and die or are culled on humane grounds, and the losses associated with an increased rate of culling of infected sheep in poor condition. Indirect

effects include the losses associated with reduced market value of infected stock and reduced pre-weaning lamb growth rates as a result of reduced milk production in ewes with mastitis (Houwers, 1990). In a recent study Pekelder *et al.*, (1994) compared the pre-weaning growth of lambs from infected and uninfected ewes under the same conditions and, following slaughter of the ewes, examined the association between growth rates and the number of lymphoid follicles within the udder. The weight gain of the lambs decreased with increasing severity of udder lesions, the growth reduction per follicle amounting to 54g over the entire pre-weaning period (80 days).

The economic consequences of infection becoming established within a flock are thus considerable. Within the U.K., established infections can result in the economic failure of individual sheep enterprises (Watt *et al.*, 1992; Milne & Gray, 1993).

1.9 The relevance of maedi

1.9.1 As an animal disease

The real and anticipated economic effects of MVV infection indicate that this disease is of immediate relevance to the U.K. sheep industry, and as such, fundamental and applied research focussed on this disease has obvious potential benefits to animal health regarding a more complete understanding of disease pathogenesis, diagnosis and control.

1.9.2 As a model for human disease

Maedi has also been proposed as a useful animal model of human lung disease, specifically LIP associated with HIV infection (Lairmore *et al.*, 1986,1988; Pétursson *et al.*, 1989,1991). A brief synopsis of LIP as it relates to human pulmonary medicine is pertinent prior to discussing the validity of such a proposal.

Human lymphoid interstitial pneumonias (LIPs), defined as the exquisitely interstitial diffuse infiltrations of the lung, predominantly by lymphocytes with varying admixtures of plasma cells and other elements (Liebow & Carrington, 1973), were first described in 1966 (Carrington & Leibow, 1966). These

Sjögrens syndrome
Thyroiditis
Myasthenia gravis
Chronic active hepatitis
Autoimmune haemolytic anaemia
Systemic lupus erythematosus
Pernicious anaemia
Diffuse lymphoid hyperplasia
HIV infection

Table 1.2 Diseases with which LIP is associated.

pneumonias are classified within the broad group of interstitial lung diseases (ILDs) which are characterized by an initial alveolitis which progresses through structural derangement of the alveolo-capillary unit to ultimate loss of the same as a result of interstitial fibrosis (Crystal *et al.*, 1981). Indeed the terms ILD and 'fibrotic lung disease' are used interchangeably (Crystal *et al.*, 1981). LIP is frequently diagnosed in association with defined clinical entities such as Sjögren's syndrome or with diseases of putative immunological origin (Teirstein & Rosen, 1988)(Table 2.1) with these being frequently associated with dysproteinaemias (Liebow & Carrington, 1973). Oleske *et al.* (1983) first described LIP associated with HIV infection (LIP-HIV) in children of mothers at high risk for the acquired immune deficiency syndrome (AIDS). Although subsequent reports identified LIP-HIV in adults (Saldana *et al.*, 1983; Grieco & Chinoy-Acharaya, 1985; Solal-Celigny *et al.*, 1985; Ziza *et al.*, 1985) this association is seen far more frequently in the paediatric population (Teirstein & Rosen, 1988). Indeed estimates of the incidence of LIP range from 30 to 50% of children perinatally infected with HIV (Jason *et al.*, 1988; Pizzo *et al.*, 1988).

The multiple associations that LIP appears to hold with various disorders and infections indicate that LIP is simply a common pathological manifestation reached via any one of a number of possible pathogenetic pathways. A common feature of human ILDs is the development of pulmonary fibrosis. Opinions differ as to whether LIP-HIV progresses to fibrosis (Teirstein & Rosen, 1988; Guillon *et al.*, 1988; Pitt, 1991). If fibrosis is allowed to progress, then the resulting 'end-stage' lung is irreversibly affected and death will ensue (Crystal *et al.*, 1981). However, as with all ILDs (Crystal *et al.*, 1981), LIP-HIV should be considered reversible as long as the remaining epithelial and endothelial cells of the interstitium have the normal basement membrane scaffolding to direct the placement of new parenchymal cells during repair (Crystal *et al.*, 1981). Indeed, therapeutic intervention is often successful in relieving clinical symptoms and improving gas exchange and radiographic appearance of LIP-HIV (Solal-Celigny *et al.*, 1985; Bach, 1987; Popa, 1988).

An overwhelming consequence of HIV infection in humans is the development of immunosuppression due primarily to the depletion of CD4 molecule expressing 'helper' T-lymphocytes. The CD4 molecule is the main surface structure susceptible to HIV-1 binding (Dalgleish *et al.*, 1984) and is expressed on 'helper' T-lymphocytes as well as on cells of the monocyte-macrophage lineage (Gartner *et al.*, 1986). In contrast, CD4 lymphocytes are not a primary target cell for infection by MVV and immunosuppression is not a significant feature of maedi-visna. These and other comparisons between ovine and human lentiviral infections are examined by Pétursson *et al.* (1991). Respecting the various arguments of Pétursson *et al.* (1991) it should be apparent that the complex multi-systemic nature of lentiviral disease makes it unlikely

that maedi-visna would fulfill all expectations as an appropriate model of human lentiviral infection. However, the broad but crucial similarities regarding viral genomic organization, the tempo of disease progression, viral persistence in the face of humoral and cell-mediated immune responses and *in vivo* restriction of replication and antigenic variation of viruses (Pétursson et al, 1989) suggest that specific components of the disease complex in sheep and humans might share common pathogenetic mechanisms. As such, the identification of components of maedi-visna holding close similarities with corresponding aspects of human lentiviral disease and which offer research opportunities unavailable in human clinical research should prove of value in extending the knowledge base relevant to both species.

In this regard maedi appears to hold several similarities with LIP-HIV. The lung pathology associated with MVV infection is morphologically similar to that described for LIP-HIV (Lairmore *et al.*, 1986). Another aspect of thoracic pathology, namely pulmonary lymph node follicular hyperplasia, is a feature of both maedi (Ellis & DeMartini, 1985) and LIP-HIV (Joshi *et al.*, 1985). LIP-HIV occurs with greatest frequency in paediatrics and commonly presents in the second to third year of life (Rubinstein *et al.*, 1988). This incubation period is similar to maedi where clinical signs may be detectable from two years of age onwards. Individuals with HIV-LIP often have a peripheral blood lymphocytosis and hypergammaglobulinaemia (Rubinstein *et al.*, 1988). Both the former (Dawson, 1980) and the latter (Molitor *et al.*, 1979) are features of maedi. With regard to the lung parenchymal interstitial infiltrate seen in maedi, there is evidence to suggest an increase in both CD4 'helper' and CD8 'suppressor' T-lymphocytes (Cordier *et al.*, 1992; Watt *et al.*, 1992). Bronchoalveolar lavage of lungs from cases of maedi demonstrates an increase in cellularity with significant increases in neutrophils and lymphocytes being consistent findings between different laboratories (Cordier *et al.*, 1992; Luján *et al.*, 1993). The bronchoalveolar lavage fluid (BALF) lymphocytosis in maedi appears to comprise primarily CD8 cells (Cordier *et al.*, 1992; Luján *et al.*, 1993). Although some debate centres around the exact phenotypic nature of the interstitial infiltrate in LIP-HIV (Pitt, 1991) there is a CD8 lymphocytosis in BALF from individuals with LIP-HIV (Solal-Celigny *et al.*, 1985; Guillon *et al.*, 1988) and also in individuals with HIV infection but without respiratory symptoms or radiographic abnormalities (Guillon *et al.*, 1988). In both maedi and LIP-HIV, macrophages show evidence of viral infection (Chayt *et al.*, 1986; Brodie *et al.*, 1992). The finding that macrophages are target cells for both HIV and MVV (Narayan *et al.*, 1982; Gartner et al., 1986) is of some consequence to the interpretation of possible pathogenetic mechanisms in lentivirus-induced LIP, in that it is reasonable to assume that the influx of lymphocytes to the lungs in LIP arises as a consequence of infection in the monocyte-macrophage cell lineage. Thus, investigations of HIV-LIP pathogenesis might benefit from experience with the MVV-LIP model in that the presence of infected CD4 molecule expressing 'helper' T-lymphocytes is not a complicating factor in the latter instance.

1.10 The role for *in vivo* investigation in maedi research

As will be appreciated from the preceding sections, considerable detail is known regarding cellular and molecular aspects of the biology of MVV ranging from the structure and organization of the viral genome through events comprising the viral life cycle to the pathological consequences of infection. A recent and concise review of the current understanding of the biology of MVV is given by Carey & Dalziel (1993).

In contrast to the above, there are no reports in the international literature that specifically define and assess the *in vivo* lung functional consequences of MVV-infection, neither has there been any attempt, beyond speculation, to interpret clinical evidence of lung dysfunction in relation to pathology i.e. to elucidate the pathophysiological mechanisms that lead to lung functional deficits. There are several reasons why such investigations should play an integral role in research into maedi pathogenesis. The first and principal reason for examining the lung functional consequences of MVV-infection and the underlying pathophysiological mechanisms is that the results of such studies would potentially provide the means of opening a functional 'window' on the disease, against which the results of parallel *in vitro* investigations could be compared. As a result, the latter investigations could be interpreted in the light of the stage of evolvement of the lung disease, thus maintaining clinical relevance to the *in vivo* situation. Currently, the only means available to stage the pathogenesis of maedi without sacrificing the animal, is by lung biopsy. However, there are considerable ethical and operational arguments against the repeated use of lung biopsy as a means of assessing lung disease pathogenesis, these arguments serving to highlight in this regard the potential value of less invasive techniques such as lung function testing, bronchoalveolar lavage or radiography. Further reasons for pursuing functional investigations in maedi concerns the fact that such studies frequently play a valuable role in identifying relevant areas for further research at either an *in vivo* functional level or cellular level, and that related human ILD research, with which to compare MVV-LIP pathogenesis, is itself heavily reliant upon clinical and functional assessment of disease. Lastly, the development and application of appropriate techniques to functionally assess the progress and prognosis of maedi is likely to yield future benefits in that such techniques will be available and may be applicable to the *in vivo* testing of hypotheses generated from *in vitro* research.

To reiterate, it is considered important that, in integrated animal disease investigations, minimally invasive methods are available at the outset to quantitatively assess the functional consequences of disease such that applied scientific research at the cellular or molecular level is generated from and can in turn be related to a quantifiable clinical abnormality and that

these methods of functional quantitation can subsequently be used or adapted to test hypotheses arising as a result of such research.

Given the potential value of lung function analysis as an investigative tool in maedi research, it is worthwhile both to consider the suitability of sheep as *in vivo* models of lung disease in general and to indicate a brief history of the recent use of lung function analysis in ovine respiratory research.

Sheep are docile, easily handled animals that are readily purchased from commercial stock markets. Housing and maintenance costs obviously depend on the specific research requirements, however these compare favourably with other species frequently used as respiratory models, such as dogs or primates. Sheep tolerate routine procedures such as blood sampling very well and do not constitute a particular anaesthetic risk. For these and other reasons the sheep is favoured as an experimental animal in surgical science (Borrie & Mitchell, 1960; Hecker, 1974). With specific respect to the respiratory system, sheep lungs are anatomically much closer to those of man than are dog, cat or monkey lungs (McLaughlin *et al.*, 1961) and when compared to dogs, sheep appear to be better suited as models for human gas exchange (Werlen *et al.*, 1984). The size of sheep lungs facilitates the application of various techniques available for the analysis of human cardiorespiratory function. Further, in sheep, time sequential analysis of the respiratory tract, including physiological, biopsy and lavage procedures, is well tolerated and can be repeated at fortnightly intervals with no procedure-related effects (Bégin *et al.*, 1981). Thus, dynamic events following natural or experimental respiratory intervention can be followed in this species.

Prior to the study of Halmagyi & Colebatch (1961) negligible data existed in the literature regarding cardiorespiratory function in sheep. The data reported by these authors included tidal volume, gas exchange, pulmonary mechanics and blood gas and acid-base values for normal anaesthetized sheep. These authors subsequently went on to utilize their experience of lung function assessment in sheep to examine cardiopulmonary dysfunction in sheep in response to various interventions of both physiological and applied interest (Colebatch & Halmagyi, 1961,1963; Halmagyi & Colebatch, 1961; Halmagyi *et al.*, 1963).

This approach whereby measurement protocols and techniques for lung function analysis are developed and refined prior to their use in the study of respiratory disease has also been followed by Bégin *et al.* (1981) in an evaluation of asbestosis in sheep (Bégin *et al.*, 1983a,b). Other groups have developed and used lung function analysis techniques in the sheep to examine airway responses (Wanner & Reinhart, 1978; Wanner *et al.*, 1979; Ahmed *et al.*, 1980; Abraham *et al.*,

1981,1983; Hutchison *et al.*, 1982, 1984; Wagner & Mitzner, 1990), lung inflammatory responses (Wheeler *et al.*, 1990a,b, 1992), lung function during anaesthesia (Coulson *et al.*, 1989, 1991), gas exchange of the lung (Werlen *et al.*, 1984; McNeil *et al.*, 1989, 1991) and physiological responses to exercise (Mundie *et al.*, 1991).

Despite a number of groups using lung function analysis techniques in sheep, reports concerning the use of these techniques to quantify pathological changes in the lungs occurring as a result of naturally occurring disease are notable by their absence. Further, few reports of pathophysiological studies which relate lung dysfunction to structural changes in the lungs in sheep exist. A notable exception is the work of Bégin *et al.* (1983a,b). However, these workers correlated lung functional changes with overall pathology as primarily assessed by lung biopsy specimens and the limitations of extrapolating data from biopsy specimens to the whole lung are intuitively obvious.

In view of the current interest in maedi both as an economically significant sheep disease and as a model for LIP-HIV, there would appear to be a valid reason for establishing techniques for quantifying lung function in naturally infected sheep such that the disease can be adequately staged in the preclinical and clinical phases. As noted above, this would in the short term provide a means of interpreting *in vitro* assays and techniques conducted on samples from infected sheep in the light of the stage of disease. In the long term, the establishment of these techniques should facilitate the *in vivo* testing of hypotheses generated from *in vitro* research at the cell biological level.

1.11 Objectives of this thesis

Following a long prepatent phase, maedi progresses to clinical disease characterised by exercise intolerance and dyspnoea. Although maedi is well characterised at a pathological level, physiological studies of the disease have not been undertaken. As a consequence, the nature of the functional abnormality that underlies the clinical disease is unknown, whether there exists measurable functional deficit in the preclinical phase of the disease is unknown and the relationship between lung pathology and functional deficit is unknown. These questions are fundamental to understanding the way in which pathological events ultimately conspire to bring about organ dysfunction and clinical disease. Further, knowledge of the way in which pathology relates to measurable lung dysfunction offers a potential means of assessing the progress and prognosis of this disease.

The objective of this thesis is therefore to define the nature of the lung functional abnormalities that characterise maedi and to determine whether relationships exist between these abnormalities and concomitant lung pathology. It is a further objective to determine, where relevant, the pathophysiological mechanisms that account for these relationships through testing of appropriate hypotheses *in vitro* or *in vivo*.

Component objectives contributing towards these aims are (a) the development and application of techniques to assess lung function in anaesthetized sheep, (b) the generation of reference values and confidence limits for lung function indices in control sheep, (c) the characterisation of the structural organisation of the normal sheep lung, and (d) the development and application of techniques pertinent to the experimental testing of hypotheses generated from observed pathophysiological relationships.

CHAPTER 2

LUNG COMPLIANCE, LUNG VOLUME AND TRANSFER FACTOR FOR CARBON MONOXIDE IN ANAESTHETISED SHEEP: NORMAL VALUES AND REPRODUCIBILITY OF MEASUREMENTS

2.1 INTRODUCTION

In human respiratory research, lung function data are frequently assessed in relation to predicted values for the subject under study. These predicted values are generated using multiple regression equations previously calculated for a population similar to that from which the subject was obtained. The best reference (independent) variables used for humans are age, sex, stature and ethnic group, with these variables accounting for 60% of the total variability about the regression lines (Cotes, 1979). In comparative respiratory research, Stahl (Stahl, 1967) demonstrated, using data available from the literature, the relationship of several respiratory variables to bodyweight over a wide range of mammalian species. Such predictions, based on values over such a large weight range, may not agree with studies on smaller groups of animals of a particular species (Stahl, 1967) where social and environmental factors may lead to physiological adaptation. Indeed in humans, bodyweight is not the anthropometric variable of choice due to large differences within populations regarding the contribution of adipose tissue and muscle in individuals (Cotes, 1979).

In sheep, where breed, age, sex and environmental and social factors may influence body condition and hence bodyweight, reference to such predictions of respiratory variables based solely on bodyweight should be cautious. For this reason, where the study of respiratory variables in a particular category of sheep is planned, predicted normal values should ideally be generated using normal sheep of similar description.

Certain routine pulmonary function tests applied to humans can show considerable intrasubject variability (Guyatt *et al.*, 1975; Pennock *et al.*, 1981; Hutchison *et al.*, 1981) and similar variability is seen when comparable lung function measurements are applied to trained conscious sheep (Begin *et al.*, 1981) and cattle (Gallivan & McDonell, 1988). Although this variability can preclude the use of these measurements to monitor individual animals over time, the use of groups of animals can overcome this limitation (Gallivan & McDonell, 1988).

Whether for ethical or procedural reasons, circumstances may dictate that lung function measurements cannot be undertaken in naive conscious animals and in these situations anaesthesia is a frequently considered alternative. However, Southorn *et al.* (1980) demonstrated that anaesthesia can contribute to intrasubject variability of pulmonary mechanics and static lung volume measurements in dogs. This variable effect of anaesthesia on pulmonary function measurements in individuals will presumably occur in other species and may limit their potential for monitoring changes in lung function in groups of animals over time.

The purpose of this study was firstly to generate regression equations relating pulmonary function variables to bodyweight for anaesthetized adult Texel sheep such that normal values could in future be generated for sheep of similar breed, age and sex and managed under similar conditions, and secondly to examine the long term reproducibility of lung function measurements obtained during anaesthesia in a group of adult Texel sheep.

2.2 MATERIALS AND METHODS

2.2.1 Animals

Sixteen adult Texel ewes (bodyweight 52 - 87kg) were used in this study (Appendix 2.1). All animals were free from clinically apparent cardiopulmonary dysfunction. Additional examinations including routine haematology, thoracic radiography (Figure 2.1), faecal examination for lungworm larvae and serological testing for presence of maedi-visna virus infection, confirmed the absence of significant cardiopulmonary dysfunction. The sheep were housed during the period of measurements.

2.2.2 Anaesthesia

Food was withheld for 12 hours prior to anaesthesia which was achieved by intravenous administration of a single bolus of thiopentone sodium (Intraval Sodium; Rhône Mérieux Ltd.) at a dose rate of 20 mg/kg bodyweight. Sheep were weighed on the morning of the procedure. Following induction the sheep were intubated using cuffed endotracheal tubes (diameter 9.5 - 10.5mm)(Figure 2.2) and placed in sternal recumbency with the head supported on a cushioned rest (Figure 2.3). Sheep were ventilated with medical air (BOC) using a mechanical ventilator (Manley MN2; Hutchinson Blease) adjusted to maintain a tidal volume of 10ml/kg bodyweight and respiratory rate of 10 breaths/min (Figure 2.4).

Figure 2.1 Thoracic radiography was used as an aid in determining that control sheep were free from significant cardiopulmonary dysfunction

Figure 2.2 Sheep were intubated using cuffed endotracheal tubes. An extended blade laryngoscope aided visualization of the larynx.

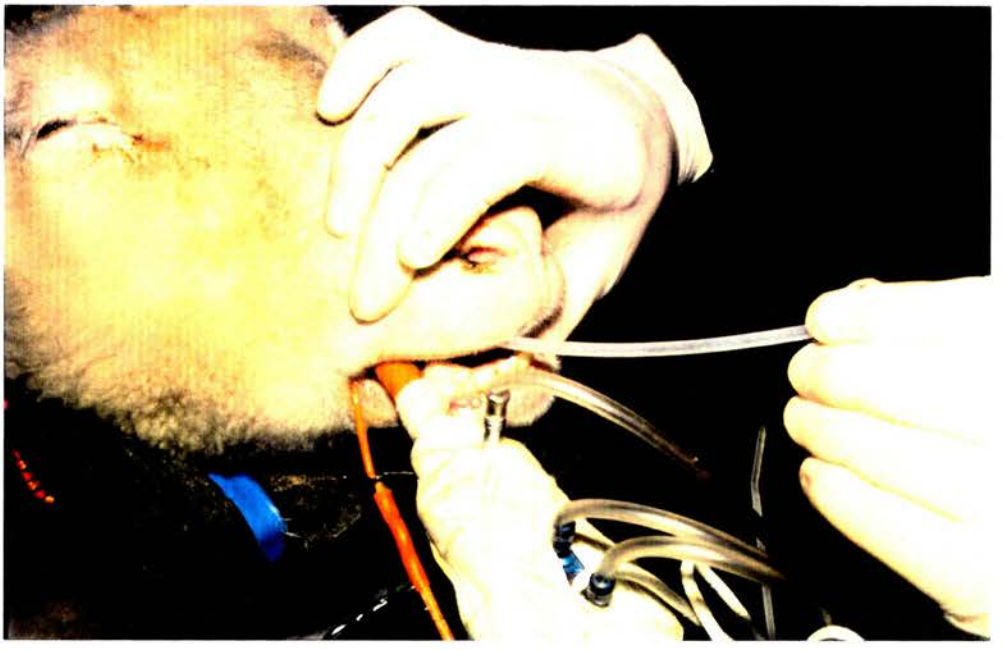


Figure 2.3 The oesophageal catheter was passed via the mouth after the sheep was intubated.

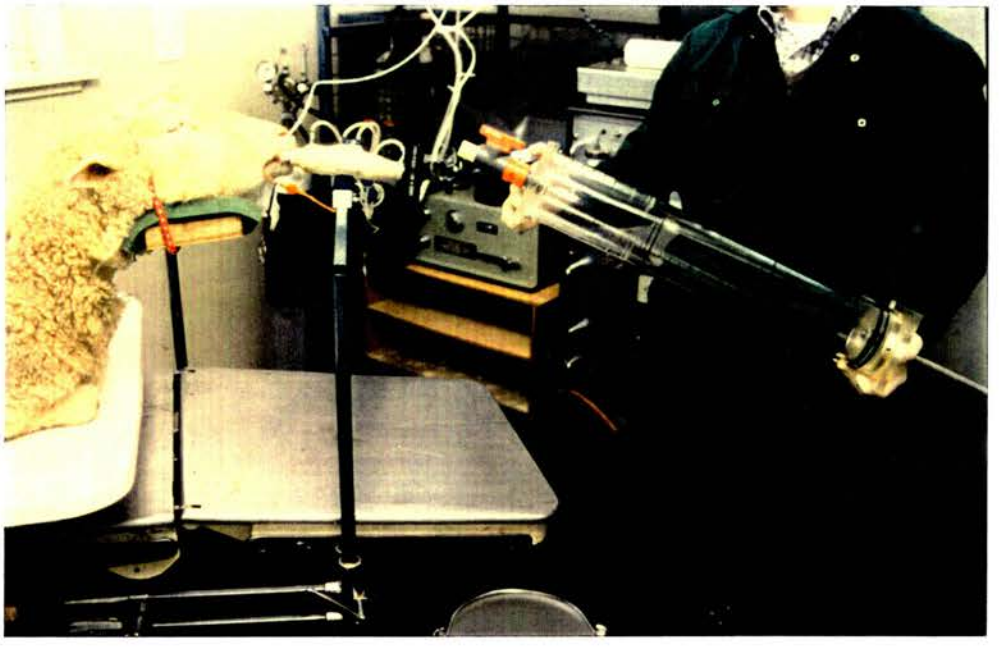


Figure 2.4 The sheep was placed in sternal recumbency with the head supported on a cushioned rest. A mechanical ventilator was used to maintain respiratory rate and tidal volume. The pneumotachograph assembly is attached to the endotracheal tube. The large calibrated syringe used to inflate the lungs with test gas mixtures is shown.

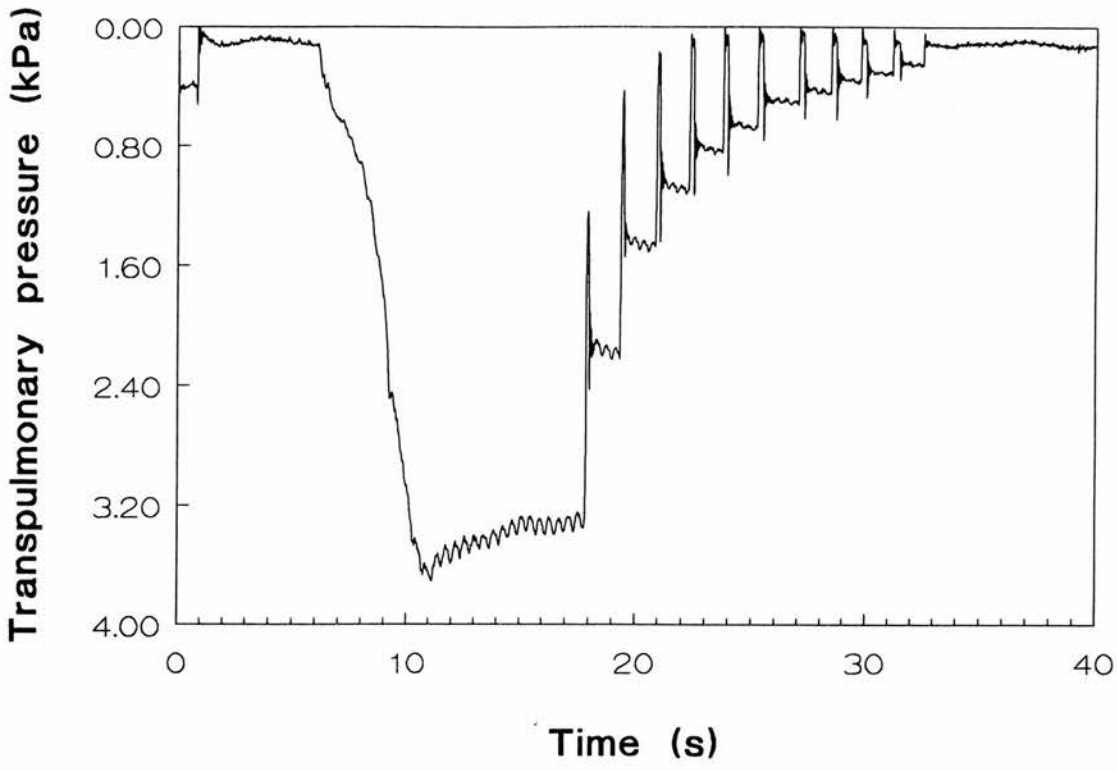


Figure 2.5 Representative transpulmonary pressure chart recording used in the calculation of static compliance (Cst)

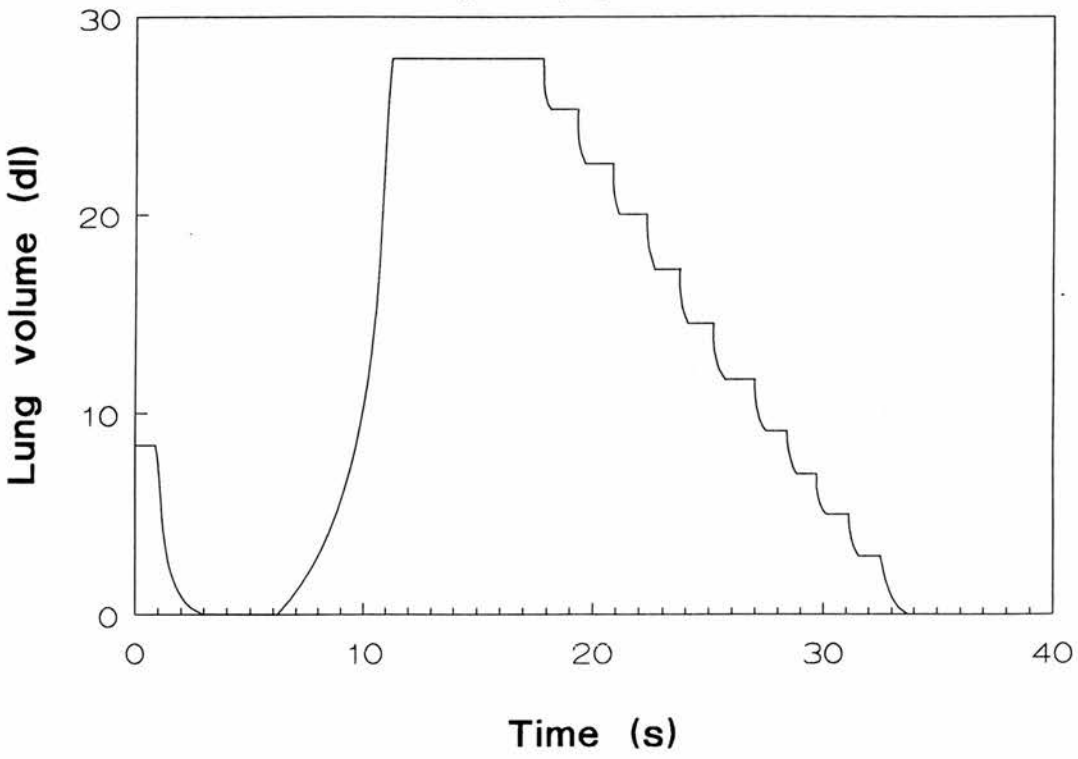


Figure 2.6 Representative transpulmonary pressure chart recording used in the calculation of static compliance (Cst)

To facilitate measurement of respiratory flows a heated pneumotachograph (Fleisch No.2; Linton Instrumentation) was connected to the end of the endotracheal tube and the pressure drop across the pneumotachograph was measured using a sensitive differential pressure transducer (CS9; Mercury Electronics) with the signal subsequently integrated to yield respiratory volumes. Changes in transpulmonary pressure (Ptp) were measured using a differential pressure transducer (CS9; Mercury Electronics). One side of this transducer was connected via a polyethylene catheter (3mm I.D., 4.5mm O.D.) to a latex balloon (length 15cm, diameter 1.5cm, thickness 0.05mm) sealed over the distal end of the catheter and placed in the caudal third of the thoracic oesophagus. Prior to each series of measurements, the pressure-volume characteristics of the balloon-catheter assembly were validated according to the method of Senterre and Geubelle (1970). The balloon was then evacuated and 2 ml of air was added (this volume being within the range of high compliance of this pressure recording system and close to the minimal relaxed volume of the balloon in air). The positioning of the balloon catheter assembly was confirmed by observing cardiogenic oscillations in the transpulmonary pressure recorder trace and by the absence of a deflection of this trace during inspiratory efforts against a closed airway (Milic-Emili *et al.*, 1964). The other side of this transducer was connected to a side port perpendicular to the airway opening.

Outputs from pressure and flow measuring devices were recorded on a strip chart recorder (Linseis L6514; Belmont Instruments). Prior to measurements being made the pressure recording system was calibrated against a water manometer and the integrated volume recording was calibrated using a 3l syringe.

2.2.3 Lung function measurements

Static lung compliance (Cst). Following cessation of spontaneous respiratory efforts (within 2-3 mins of commencing mechanical ventilation in every case) the sheep were disconnected from the ventilator and allowed to passively exhale to functional residual capacity (FRC). The lungs were then actively inflated to Ptp = 3kPa using the 3l calibrated syringe filled with room air (Figure 2.4) and immediately thereafter allowed to passively deflate to FRC. This procedure was repeated twice to stabilize lung volume history prior to measurements of static compliance being made. The volume of the lungs at Ptp = 3kPa was defined as total lung capacity (TLC). To measure Cst, the lungs were again inflated to TLC, however the passive exhalation was interrupted in a stepwise fashion by occluding the airway opening 8-15 times for 2-3 second intervals (Figure 2.5-2.6; Facing page). By plotting Ptp against expired volume a static deflation curve was obtained. The slope of the linear portion of the curve from FRC to 40% of the way from FRC to TLC was determined by least squares linear regression and this value taken as Cst, expressed in units of l/kPa. To achieve adequate data points for the least squares regression, the measurement procedure outlined above was

repeated twice with pooling of data points such that a single 'within procedure' value of Cst for each sheep was obtained.

Effective alveolar volume ($V_{A,eff}$) and *transfer factor for carbon monoxide* ($T_{L,CO}$). $V_{A,eff}$, using a single-breath helium dilution technique, and $T_{L,CO}$, using a forced single-breathhold technique, were measured concurrently as part of the same procedure. Following a period of mechanical ventilation the sheep were disconnected from the ventilator and allowed to passively exhale to FRC. The 3l calibrated syringe was then used to inflate the lungs to Ptp = 3kPa with a carbon monoxide and helium in air gas mixture (14% helium, 0.3% carbon monoxide, 85.7% air; BOC)(Figure 2.4). A valve at the airway opening was closed and this gas mixture was held in the lungs for a period of 10-12 seconds. The valve was then opened momentarily to allow washout of deadspace before the remaining gas in the lungs was collected in an evacuated rebreathing bag. The concentration of helium in this expired alveolar gas sample was measured using a katharometer (Resparameter Mk 4; P.K. Morgan) and $V_{A,eff}$ in litres, i.e. the alveolar volume at which the breath was held minus the dead space of the airways and equipment was calculated as follows:

$$V_{A,eff} = 1.05 \cdot (Vsyr - VD,an - VD,eq) \cdot (HeI/HeE)$$

where Vsyr = volume used to inflate lungs to Ptp = 3kPa, VD,an = anatomic dead space, VD,eq = instrumental dead space, HeI = helium concentration in the syringe and HeE = helium concentration in the expired sample (Quanjer *et al.*, 1983). Instrumental deadspace was calculated from internal dimensions and the anatomic deadspace was estimated using the equations of Stahl (1967) which relate respiratory variables to bodyweight in a range of species (Appendix 2.2).

Single-breath transfer factor for carbon monoxide ($T_{L,CO,'sb'}$) was calculated as follows:-

$$T_{L,CO,'sb'} = 53.6 \cdot V_{A,eff} \cdot t^{-1} \cdot \log10 (COI \cdot HeE \cdot COE^{-1} \cdot HeI^{-1})$$

where t = time of breathholding in seconds and HeE and COE were alveolar concentrations, and HeI and COI syringe concentrations of helium and carbon monoxide respectively (Cotes, 1983). Time of breathholding was calculated according to the recommendations of the Epidemiology Standardization Project (ESP)(Ferris, 1978)(Appendix 2.3). A blood sample was taken during the period of anaesthesia and haemoglobin concentration measured using an automated analyser (System 9000; Baker Instruments, PA, USA). $T_{L,CO,'sb'}$ values were then adjusted to a standard haemoglobin concentration of 14.6 g/dl according to the method of Cotes (Cotes, 1979)(Appendix

2.4). The linearity of the helium and carbon monoxide meters was assessed using a modification of the technique described by Quanjer et al. (1983) (Appendix 2.5). $T_{L,CO,'sb'}$ is expressed in units of mmol/min/kPa. Since the transfer factor is positively correlated with the lung volume at which the measurement is made, it was also expressed per litre of alveolar volume i.e. $T_{L/VA}$. The mean value of three determinations of $V_{A,eff}$ and $T_{L,CO,'sb'}$ was calculated and used in the statistical analyses. The average duration of the completed series of measurements was approximately 20 minutes.

2.2.4 Experimental design and statistical analysis

Long term reproducibility of the lung function data was examined by repeating measurements in the same group of 9 sheep at monthly intervals over a period of 5 months. A two-way mixed model analysis of variance without replication was used to estimate the between day variability of lung function variables for the group. In this analysis, the individual sheep are considered the random factor and the time dimension a fixed treatment effect (Sokal & Rohlf, 1981). A Kolmogorov-Smirnov test was used to test for normal distribution of the pooled error terms in each anova and a Bartlett's test was used to check for homogeneity of variances between samples in each anova (Sokal & Rohlf, 1981). The coefficient of variation (CV) for the repeated measurements of each variable in individual sheep was also calculated.

The relationship of individual lung function variables to bodyweight was examined for all 16 sheep. Lung function and bodyweight measurements were made at least three times on each sheep with the interval between measurements being between 30 and 77 days. The data from a total of 70 procedures were used in the analysis. Regression analyses based on linear, polynomial and allometric regression equations were executed to determine the relationship between bodyweight (the independent variable) and the lung function data (the dependent variable). Using the most statistically significant regression equations, 95% prediction intervals for individual lung function variables were calculated over the range of bodyweights studied.

2.3 RESULTS

Results of Cst, $V_{A,eff}$, $T_{L,CO,'sb'}$ and $T_{L/VA}$ measurements made at monthly intervals are given in appendix 2.6. The error terms in each anova were normally distributed (Appendix 2.7) and the variances were homogenous (Appendix 2.8). Long term variability of Cst, $V_{A,eff}$, $T_{L,CO,'sb'}$ and $T_{L/VA}$ are illustrated in Figures 2.7-2.10 (overleaf).

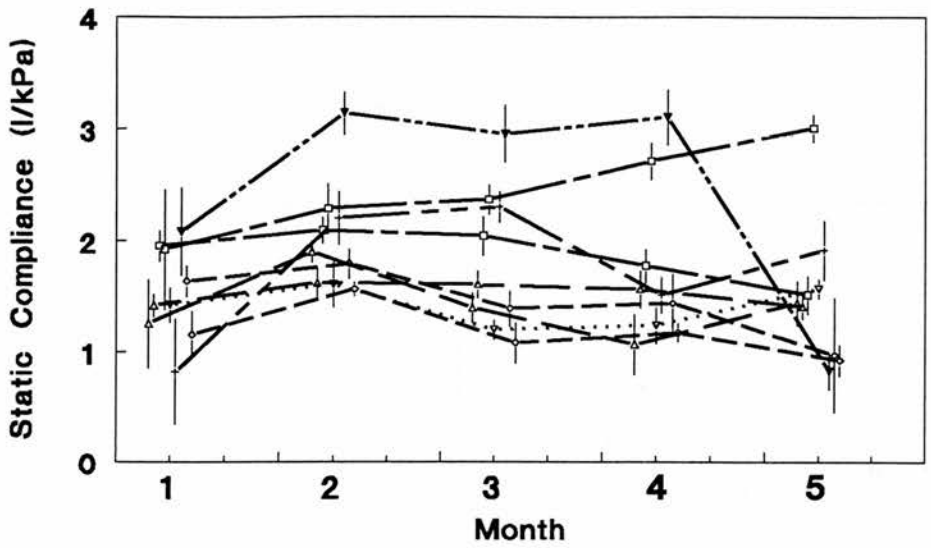


Figure 2.7. Long term variability of Cst measurements made in nine sheep at monthly intervals over a period of 5 months. Error bars in the upper graph represent the 95% confidence limits for the slope of the regression line between pressure and volume measurements.

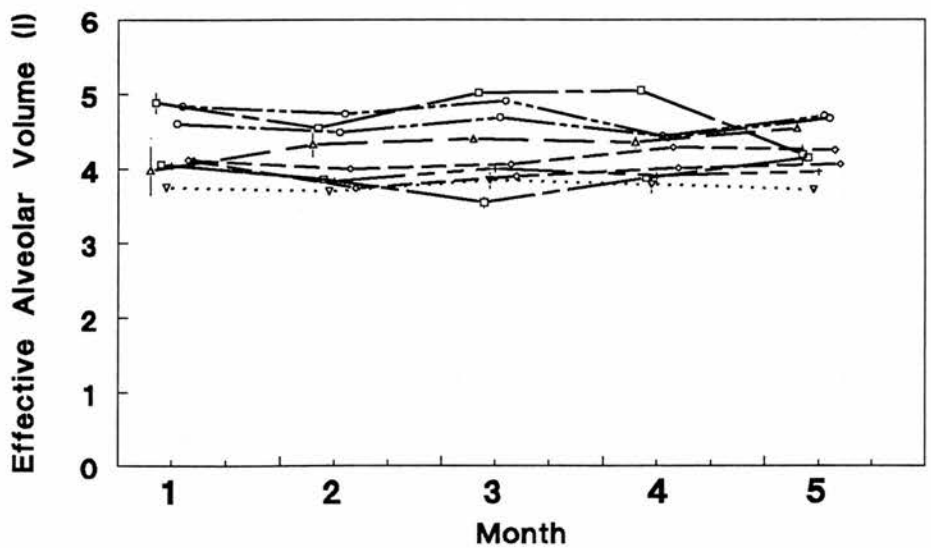


Figure 2.8. Long term variability of $V_{A,eff}$ measurements made in nine sheep at monthly intervals over a period of 5 months. Error bars represent ± 1 S.D..

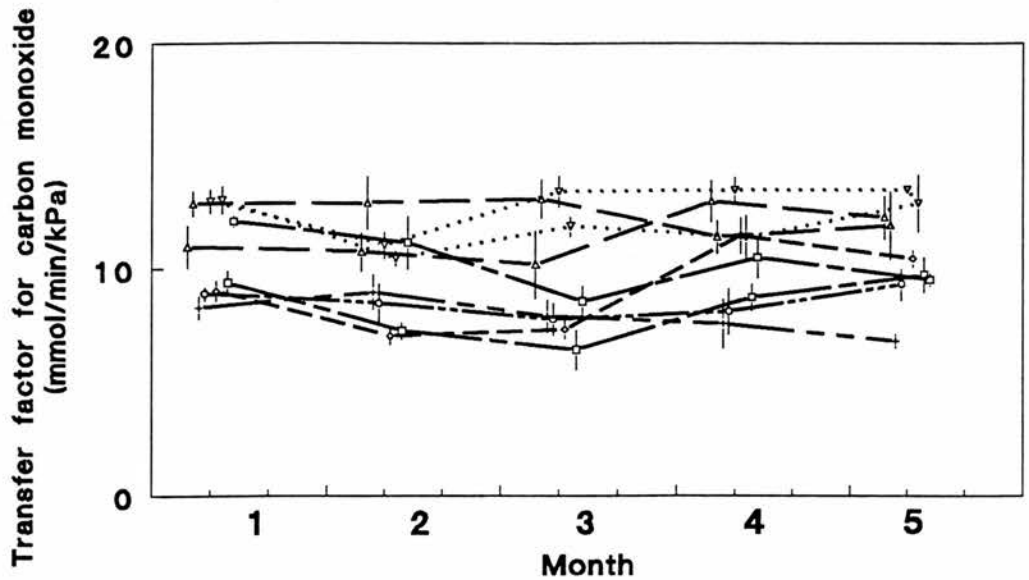


Figure 2.9. Long term variability of $T_{L,CO,'sb'}$ measurements made in nine sheep at monthly intervals over a period of 5 months. Error bars represent ± 1 S.D..

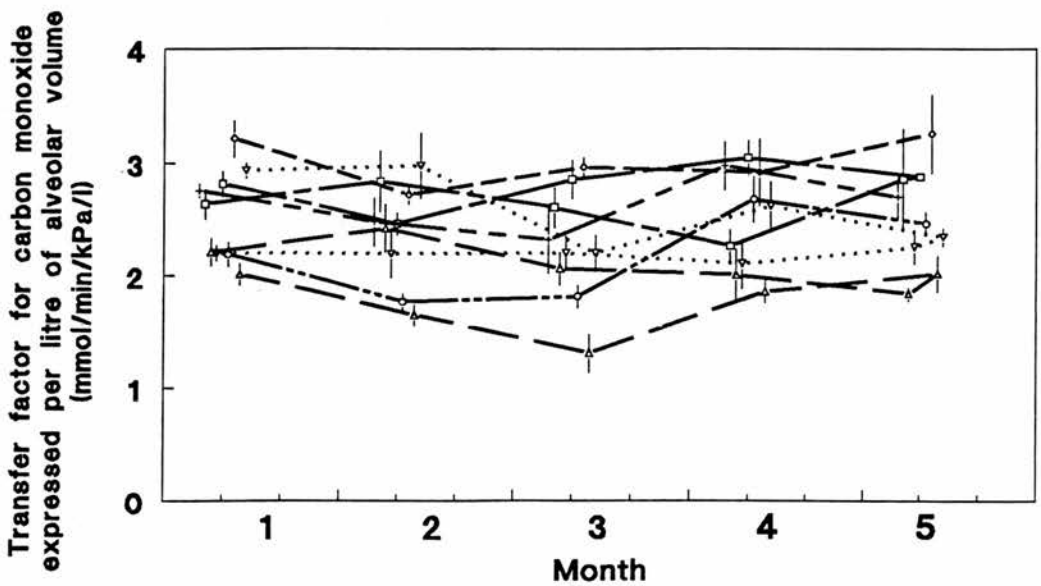


Figure 2.10. Long term variability of $T_{L/VA}$ measurements made in nine sheep at monthly intervals over a period of 5 months. Error bars represent ± 1 S.D..

Coefficients of variation for repeated measurements of each variable in individual sheep are illustrated in Figure 2.11.

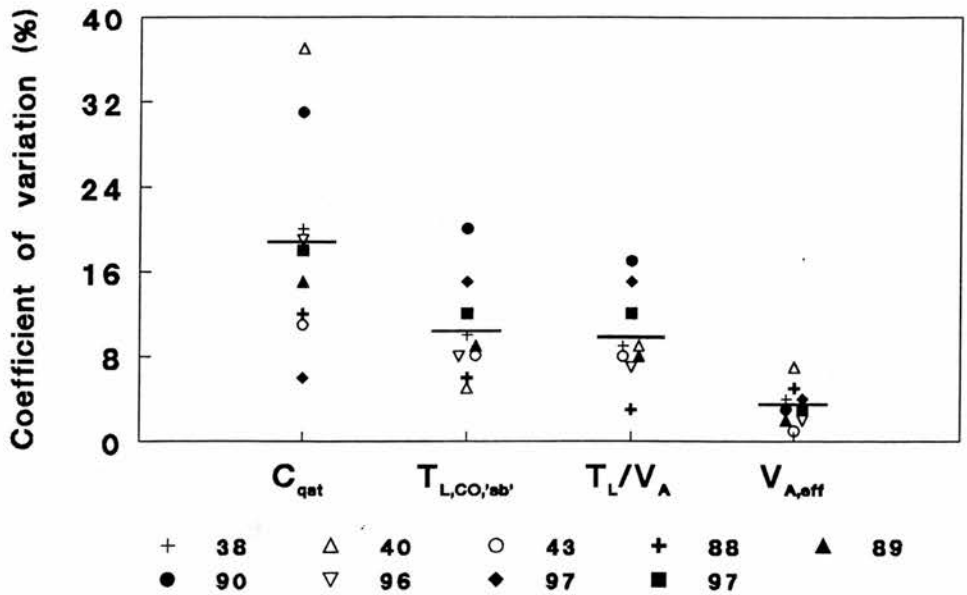
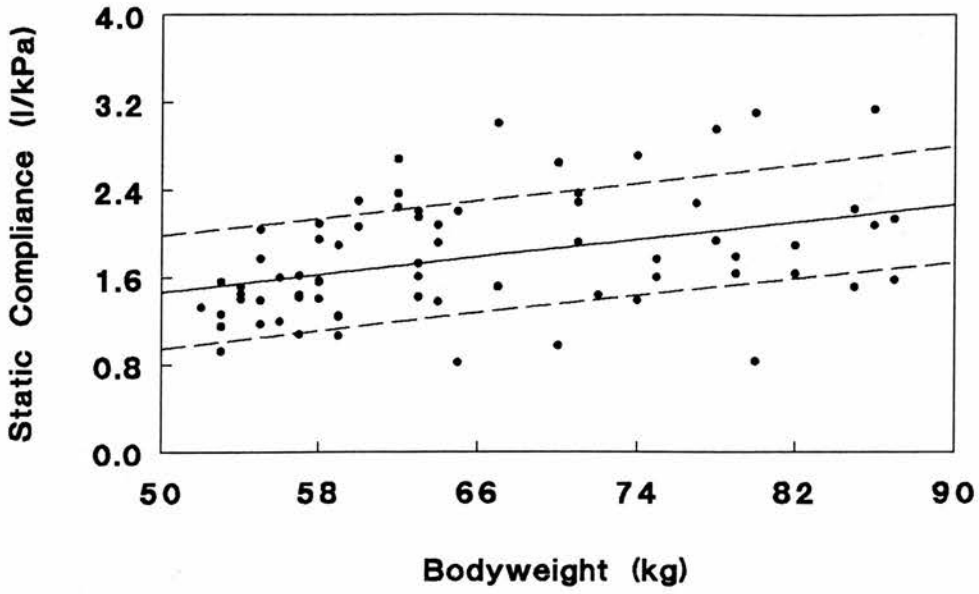


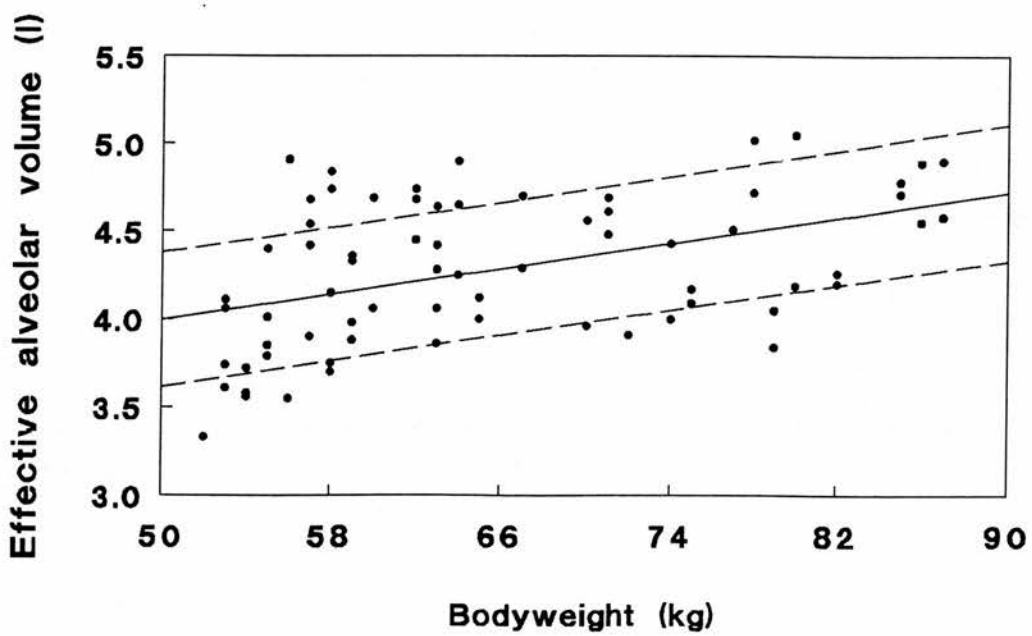
Figure 2.11 Individual coefficients of variation for repeated measurements of Cst, $V_{A,eff}$, $T_{L,CO,'sb'}$ and $T_{L/VA}$ made in nine sheep at monthly intervals over a period of 5 months. The horizontal bars represent the mean values.

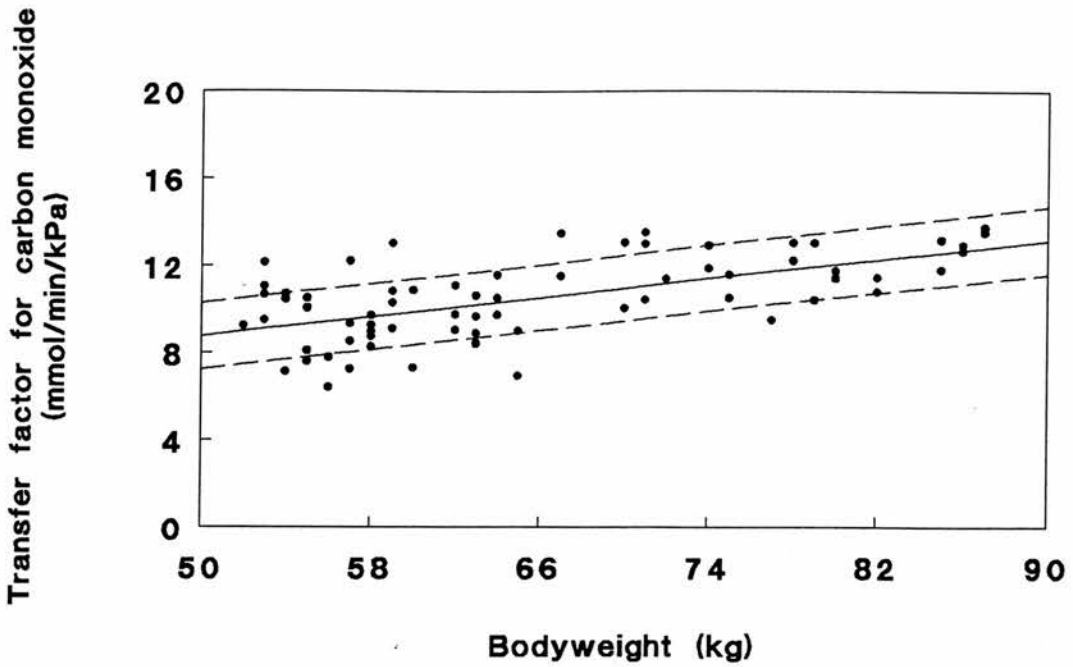
The average intrasubject CV for the repeated measurements was 18.8 $\pm$ 9.23 (mean $\pm$ standard deviation) for Cst, 10.3 $\pm$ 4.45 for $T_{L,CO,'sb'}$, 9.8 $\pm$ 4.02 for $T_{L/VA}$ and 3.4 $\pm$ 1.71 for $V_{A,eff}$. The results of the analyses of variance are shown in appendix 2.9. There were no significant differences between days for the group for any of the lung function variables although between sheep differences were highly significant (Appendix 2.9).

Data from the reproducibility analysis were included in the data set for regression analysis with bodyweight as the independent variable. In addition, results of Cst, $V_{A,eff}$, $T_{L,CO,'sb'}$ and $T_{L/VA}$ measurements made in sheep not included in the reproducibility analysis were included in the regression analysis (Appendix 2.10). A significant relationship was found between all of the measured variables and bodyweight. These relationships are illustrated in Figures 2.12-2.15 (overleaf).

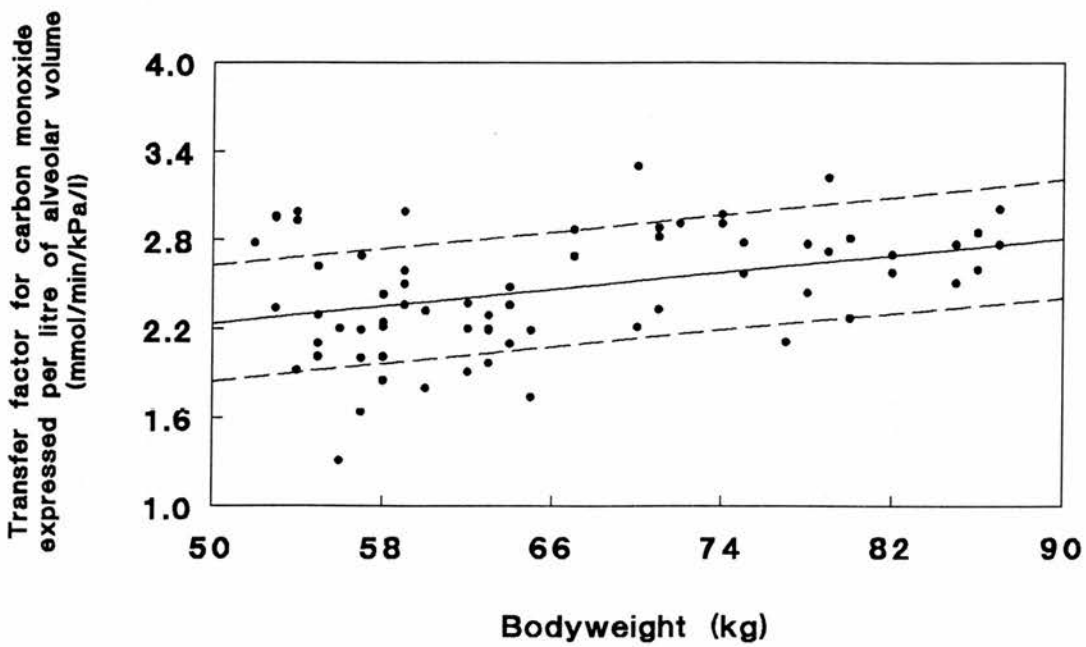


Relationship between Cst (l/kPa) and $V_{A,eff}$ (l) and bodyweight (Figure 2.12 above & 2.13 below). Repeated measurements were made in 16 sheep. Solid lines depict the regression equations that best represent the relationship between the dependent variables and bodyweight. Dotted lines represent the 95% prediction intervals for the dependent variables in individual subjects.





Relationship between $T_{L,CO,'sb'}$ (mmol/min/kPa) and $T_{L/VA}$ (mmol/min/kPa./l) and bodyweight (kg) (Figure 2.14 above & 2.15 below). Repeated measurements were made in 16 sheep. Solid lines depict the regression equations that best represent the relationship between the dependent variables and bodyweight. Dotted lines represent the 95% prediction intervals for the dependent variables in individual subjects.



The most significant regression equations were linear for all variables and these equations, together with the variance ratio (F), the determination coefficient (r^2) and degree of significance of the variance ratio (P) are shown in Table 2.1 below.

Variable	Unit	Regression Equation	F	r^2	P
Cst	(l/kPa)	0.460+0.0201xBW	11.72	0.149	0.001
$V_{A,eff}$	(l)	3.09+0.0181xBW	17.53	0.207	<0.001
$T_{L,CO,'sb'}$	(mmol/min/kPa)	3.27+0.110xBW	40.79	0.378	<0.001
$T_{L/VA}$	(mmol/min/kPa/l)	1.52+0.0143xBW	10.47	0.135	<0.005

Table 2.1

2.4 DISCUSSION

The predictions of Stahl (1967) encompassed the variables measured in this study and these variables were shown to be positively correlated with bodyweight. Figures 2.16-2.19 compare regression lines generated from Stahl's power law predictions for lung compliance, total lung capacity, transfer factor and transfer factor/TLC with regression lines generated in this study.

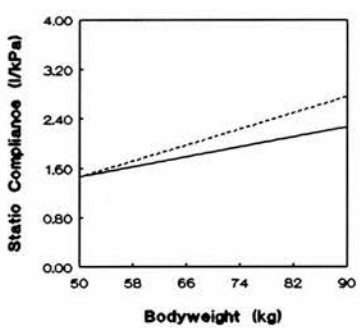


Figure 2.16

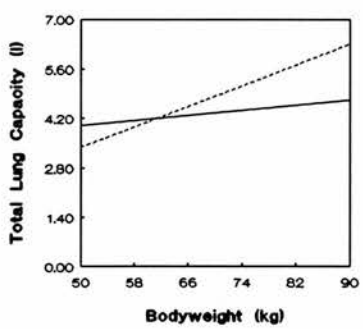


Figure 2.17

Figures 2.16-2.17 & 2.18-2.19 (overleaf). Predicted regression lines relating Cst, $V_{A,eff}$, $T_{L,CO,'sb'}$ and $T_{L/VA}$ to bodyweight. The dashed lines are from the power law formulae of Stahl (1967) generated using data collected from the literature and featuring a wide range of mammalian species. The solid lines are from the present study (Table 2.1).

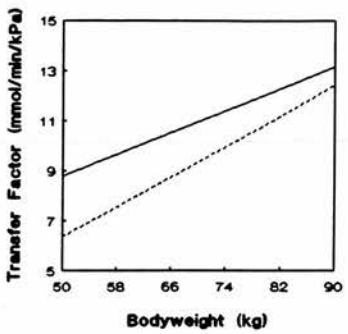


Figure 2.18

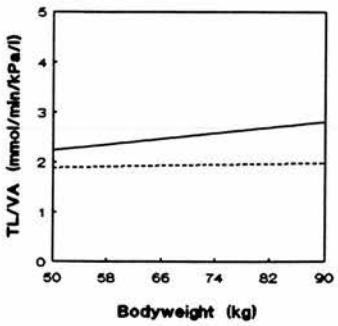


Figure 2.19

A comparison of the slope of the respective regression lines for TLC indicates that this variable remains almost constant for adult Texel sheep over the studied weight range and this observation contrasts markedly with Stahl's predictions (1967). One explanation for this anomaly would be that rather than reflecting an increase in skeletal dimensions, greater weight in these sheep reflects an increase in adipose and perhaps muscle tissue. That this is indeed likely is evidenced by the reduction in specific effective alveolar volume ($sV_{A,eff}$) i.e. alveolar volume expressed per unit bodyweight, with increasing bodyweight in these sheep (Figure 2.20). The ratios of various lung volumes to bodyweight are relatively constant and size-indepedent i.e. they do not change appreciably between species (Stahl, 1967). The reduction in $sV_{A,eff}$ with increasing bodyweight indicates that overfeeding of sheep can deleteriously alter the normally optimal relationship between body size and lung functional indices. This is a recognised concept in humans (Schoenberg *et al.*, 1978) where increased muscularity or obesity have opposite effects on ventilatory function.

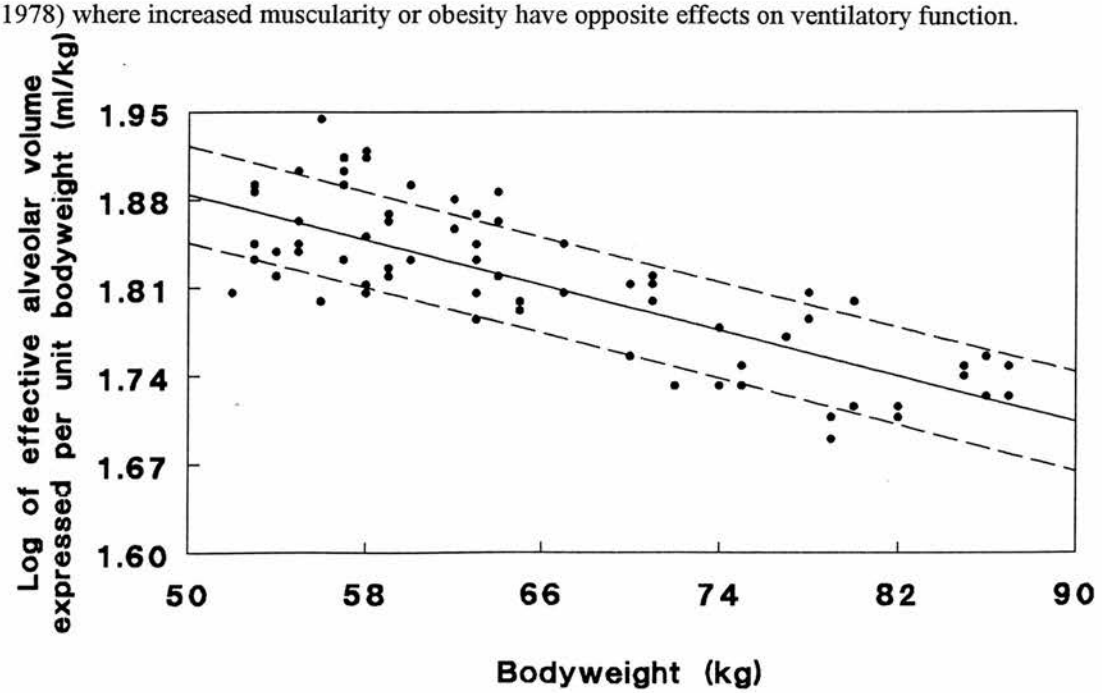


Figure 2.20 Relationship between log $sV_{A,eff}$ (ml/kg) and bodyweight (kg).

Given the volume dependence of Cst, $T_{L,CO,'sb'}$ and $T_{L/VA}$ measurements, the reduction in $sV_{A,eff}$ with increasing bodyweight will also contribute to relative slope discrepancies between Stahl's predicted regression lines and those generated in this study for these variables. Absolute differences between respective regression lines, particularly with respect to transfer factor measurements, will reflect differences in methodologies used (Robinson *et al.*, 1972) with Stahl accumulating data from many studies using variable techniques.

One previous study of respiratory variables in anaesthetized sheep of bodyweight 42.6 $\pm$ 4.7 kg (mean $\pm$ S.D.) reported a value of dynamic lung compliance of 106 $\pm$ 31 ml/cmH_20 i.e. 1.08 $\pm$ 0.316 l/kPa (mean $\pm$ S.D.)(Halmagyi & Colebatch, 1961). This value is slightly lower than anticipated from the appropriate regression equation in table 2.1 (1.32 l/kPa for a sheep of weight 43 kg), however given a ratio of dynamic compliance to Cst of 0.9 (Begin *et al.*, 1981) simple multiplication yields a value of 1.2 l/kPa. The remaining discrepancy may simply reflect progressive lung collapse through atelectasis of dependent lung units in the study of Halmagyi & Colebatch (1961). In the present study, lung volume history is frequently standardized during a relatively short period of anaesthesia, thus progressive lung collapse is unlikely. In the present study, thiopentone was administered as a single bolus dose, and as such, the level of anaesthesia would vary during the procedure. As a consequence, static lung volumes and mechanical and gas exchange properties of the lungs would also vary (Southorn *et al.*, 1980). This variation will necessarily be included in the measurements made, however the adoption of a standardized dosage regime and procedural protocol will have helped to minimize such between-procedure variation.

The observed regression equations relating bodyweight to lung function variables should prove valuable in predicting normal values for sheep of similar breed, age, sex, weight and conformation to those used in the present study and studied under identical conditions. Certainly , the observed discrepancies between predictions based on power law formulae derived from multi-species comparisons (Stahl, 1967) and those of the present study validate this approach. The 95% prediction intervals were calculated using the estimated standard deviation of the dependent variables over the range of weights studied (Gardner & Altman, 1989). These intervals serve to define the limits of normality for measurements of pulmonary function variables made in individual sheep with similar characteristics to those studied. The use of the standard deviation as a measure of variability within a population is appropriate where the data is homoscedastic, i.e. the variation is independent of the magnitude of the value measured. There are however, inadequate data points collected in this study to accurately determine whether lung function data in sheep is homo- or heteroscedastic.

A knowledge of the intrasubject variation over a period of time is essential if time sequential changes in pulmonary function values for individuals are to be accurately interpreted. In human respiratory research, data is often treated as heteroscedastic and reproducibilty expressed in terms of the coefficient of variation (CV). By expressing the long-term variability for repeated measurements in terms of the CV, knowledge of what would constitute a significant change in an individuals' lung function can be gained. Given the calculated average CV's for repeated measurements in normal anaesthetized sheep (Figure 2.11), it can be predicted (Pennock *et al.*, 1981) that significant ($p<0.05$) month-to-month changes are approximately 30.8% in the Cst, 16.9% in the $T_{L,CO,'sb'}$, 16.1% in the $T_{L/VA}$ and 5.6% in the $V_{A,eff}$ measurements. Previous reports have demonstrated similar intrasubject variability of lung function measurements in humans ((Guyatt *et al.*, 1975; Hutchison *et al.*, 1981), ponies (Derksen *et al.*, 1982), cattle (Kiorpes *et al.*, 1978; Gallivan & McDonell, 1988) and goats (Bakima *et al.*, 1988) (Table 2.2) and illustrate the problems of interpreting lung function in individuals over time.

		Measurement CV			
	Species	Cdyn	Cst	FRC	TLC
Guyatt *et al.*, 1975;	Human	24.9	19.4		
Hutchison *et al.*, 1981	Human			9*	
Derksen *et al.*, 1982	Equine	39	28		13.2
Gallivan & McDonell, 1988	Bovine	20			
Kiorpes *et al.*, 1978	Bovine	26		22	
Bakima *et al.*, 1988	Caprine	18.97			
Present study	Ovine		18.8		3.4

Table 2.2 Reproducibility of lung function measurements in different species. Time between measurements varied from days to months according to study protocols.
* indicates that the data was estimated from a chart.

For all of the measured variables, the variation between repeated measurements on the separate days for the group was insignificant, whereas variation between individual sheep was highly significant. Collective lung functional measurements on groups (n=9) of normal anaesthetized sheep do not therefore change significantly over time. Gallivan & McDonell (1988) in repeated analyses on 4 conscious adult cows found significant differences between days for the group for respiratory frequency and inspiratory and expiratory times and concluded that a control group should be used during any long-term study on small groups of animals.

Knowledge of both the predicted normal ranges for the variables Cst, $V_{A,eff}$, $T_{L,CO,'sb'}$ and $T_{L/VA}$, and awareness of the long-term reproducibility of measurements of these variables in anaesthetized adult Texel sheep will allow more accurate interpretation, on a research basis, of respiratory disease in similar animals.

2.5 SUMMARY

Measurements of static compliance (Cst), effective alveolar volume ($V_{A,eff}$) and single-breath transfer factor for carbon monoxide ($T_{L,CO,'sb'}$) were completed in 16 normal, anaesthetized, adult Texel ewes. Regression equations were computed for these variables as a function of bodyweight and the optimal equations selected. The 95% prediction intervals for the equations were calculated such that normal lung function in similar sheep could be accurately predicted.

The long-term reproducibility of these measurements was assessed in 9 sheep, measured at monthly intervals over a period of 5 months. Although measurements made in individual sheep were often highly variable, the variation between repeated measurements on the separate days for the group was insignificant.

CHAPTER 3

EXPONENTIAL ANALYSIS OF THE PRESSURE-VOLUME CHARACTERISTICS OF OVINE LUNGS

3.1 INTRODUCTION

Static lung compliance, analysed as the lung volume change per unit pressure change in the tidal volume range ($\Delta V/\Delta P$), is often assumed to be an index of lung elastic recoil, and is frequently used as such. Unfortunately, when comparing animals of different body size or comparing animals with varying stages of lung disease, the above assumption is subject to qualifications which limit the value of this measurement.

Firstly, variation in the end-expiratory level will alter the portion of the curve over which the compliance measurement is made i.e. the measurement of compliance will depend on the end-expiratory lung volume, and secondly, compliance values intuitively depend on the inflatable volume of the lungs themselves. Thus, accurate interpretation of compliance values demands both a knowledge of the lung volumes over which the measurement is made and a perception of the predicted normal value for the animal concerned. These latter requirements place an extra burden on measurement protocol and rely heavily on the accuracy of prediction equations based on anthropometric data.

An alternative means of analysing pressure-volume curves was pioneered by Salazar and Knowles (1964) who used an exponential equation to describe the pressure volume curve of human adults. Glaister *et al.* (1973) employed the same principle to describe the pressure volume curves of excised dog and monkey lungs. The basis behind this method of analysis, which utilizes all the pressure and volume data points collected, has been developed and applied to the analysis of pressure-volume curves from both healthy adults (Pengelly, 1977; Colebatch *et al.*, 1979a,b; Gibson *et al.*, 1979; Knudson and Kaltenborn, 1981; Colebatch and Ng, 1986) and those with respiratory disease (Gibson *et al.*, 1979; Colebatch *et al.*, 1985; Thompson and Colebatch, 1989). The exponent K of the fitted exponential equation is not influenced by absolute values of pressure or volume and only delineates the relative changes of one with respect to the other. In other words, K is an index of pulmonary distensibility and appears not to suffer from the aforementioned limitations of compliance measurements.

Due to structural and functional similarities between the lungs of sheep and those of humans, this species has frequently been used to model human lung disease. Although pulmonary mechanics measurements are frequently included in such studies, there has, to the author's knowledge, been only one attempt to fit an exponential equation to the normal ovine pressure-volume curve (Schroter, 1980). In view of the relative advantages of expressing pulmonary distensibility in this way this study sought to determine whether the ovine pressure-volume curve could be adequately described by an exponential equation and to report normal values of *K* for this species such that in future the distensibility of the ovine lung could be accurately assessed in disease and in relation to other species.

3.2 MATERIALS AND METHODS

3.2.1 Animals

15 adult Texel and 3 Texel-cross female sheep (median bodyweight 63.5; range 54 to 82 kgs) were used in this study (Appendix 3.1). The sheep were fed on a diet of proprietary concentrate and hay and were assessed to be free of significant cardiopulmonary dysfunction on the basis of a thorough clinical examination.

3.2.2 Anaesthesia

Anaesthetic protocol was as described in chapter 2 (section 2.2.2).

3.2.3 Measurements

The precise methodology employed to measure transpulmonary pressures and respiratory flows and volumes has been described previously (Chapter 2; section 2.2.3).

Absolute values for effective alveolar lung volume ($V_{A,eff}$) were measured using a single-breath helium dilution technique as previously described (Chapter 2, section 2.2.3).

As described in chapter 2, section 2.2.3, pressure-volume data were obtained after three forced inflations to TLC. The lungs were again inflated to TLC, however a stepwise deflation was achieved by intermittently interrupting the airway opening for 2-3 second intervals.

Three collections of lung volume and pressure-volume data were made on each animal during the period of anaesthesia. To obtain absolute values for pressure-volume curves the expired volumes were subtracted from the $V_{A,eff}$ values determined immediately prior to collection of pressure-volume data. Static lung compliance (Cst) was calculated as the slope of the pressure-volume curve between the end-expiratory level and this level plus 400mls.

3.2.5 Statistical analysis

Exponential analysis of pressure-volume curves: An exponential curve of the form

$$V = Vmax - Ae^{-K \cdot P}$$

was fitted to the data from each sheep where P is the static recoil pressure, Vmax represents the volume asymptote, A is the difference between Vmax and the intercept on the volume axis and K represents the slope of the P-V curve and therefore defines its shape. The method of Pengelly (1977) was used to determine the exponential function that best fitted the data (Appendix 3.2). Briefly, by expressing the difference between Vmax and the measured volume as a fraction of Vmax and taking the natural logarithm of this value a straight line plot with P is obtained. Least squares regression is then used to solve for slope and intercept. However, since Vmax is unknown at the outset an iterative regression is conducted until values of the coefficient of determination (r^2) are maximized. Curves were fitted both over the whole data range and over a restricted data range in which the data points less than 50% of TLC were excluded from the analysis. In addition to assessing r^2, curves were evaluated visually for goodness of fit and both a sign test for distribution of the data about the fitted regression line and a runs test to determine whether the order of the data was random ($P<0.05$) were performed (Gibson *et al.*, 1979). Associations between variables were assessed using the Spearman rank correlation coefficient (r_s).

3.3 RESULTS

The results of Cst measurements and curve fitting analysis over the whole data range for 18 sheep are shown in appendix 3.3. The summary results are shown in table 3.1. The pressure-volume data from two sheep with exponential curves fitted are presented in Figure 3.1. These examples represent the opposite ends of the spectrum in terms of goodness of fit as assessed by r^2.

Measurement	Median	Range
Data points/animal	41	26 - 48
K	1.456	1.091 - 2.119
A (ml)	2542	1590 - 3252
Vmax (ml)	4168	3277 - 4896
r^2	98.74	96.11 - 99.74
Cst (l/kPa)	2.050	1.131 - 3.698

Table 3.1. Summary results from Cst measurements and exponential pressure-volume curve fitting analysis of the form $V = Vmax - A \cdot e^{-K \cdot P}$ in 18 normal sheep.

In general the regression lines appeared to closely fit the pressure-volume data on visual inspection. Although only one pressure-volume curve failed the sign test ($P<0.05$)(Appendix 3.4.1), all but two failed the runs test ($P<0.05$)(Appendix 3.4.2) indicating that there were systematic deviations from the fitted regression lines in almost all the sheep i.e. the order of these deviations was not random. The nature of these deviations is demonstrated in a plot of the residuals against pressure (Fig. 3.2, overleaf) (with pressures expressed as percentages of the range between the minimum (0%) and maximum (100%) pressure recorded for each set of data points). The most obvious systematic deviations from the fitted regression lines occurred when transpulmonary pressure was

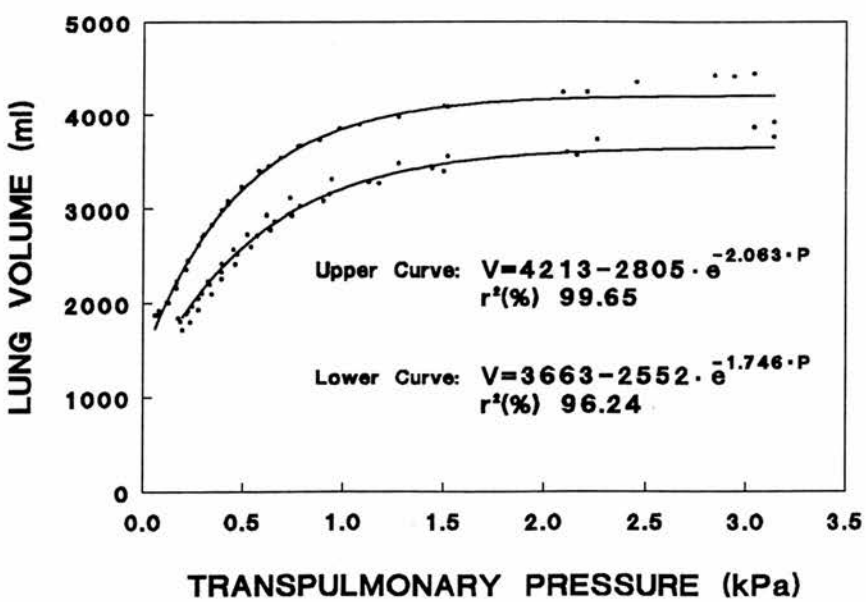


Figure 3.1 Pressure-volume data from two normal sheep. The two curves are representative of the extremes of quality of fit of data.

maximal with measured volumes being greater than the regression would seem to predict. Curves fitted data well at lower volume limits. Curve fitting over the restricted data range improved the quality of fit (as assessed by r^2) in only one instance (Appendix 3.5). In the remaining sheep, fitting a curve to the restricted data range led to a slight reduction in r^2 (median 98.34;range 95.42-99.39)

and K (median 1.348;range 0.820-2.098), and an increase in Vmax (median 4202; range 3468 - 4896) (Appendix 3.6).

The iterative regression was repeated on the pooled data from all sheep with volume expressed as percent of Vmax. The exponential equation:

$$V/Vmax\ (\%) = 100 - 56.7 \cdot e^{-1.37 \cdot P}$$

was found to best fit this pooled data. The relationship between transpulmonary pressure and lung volume expressed as percent predicted Vmax is depicted in figure 3.3. with the shaded area representing the 95% confidence interval for normal sheep.

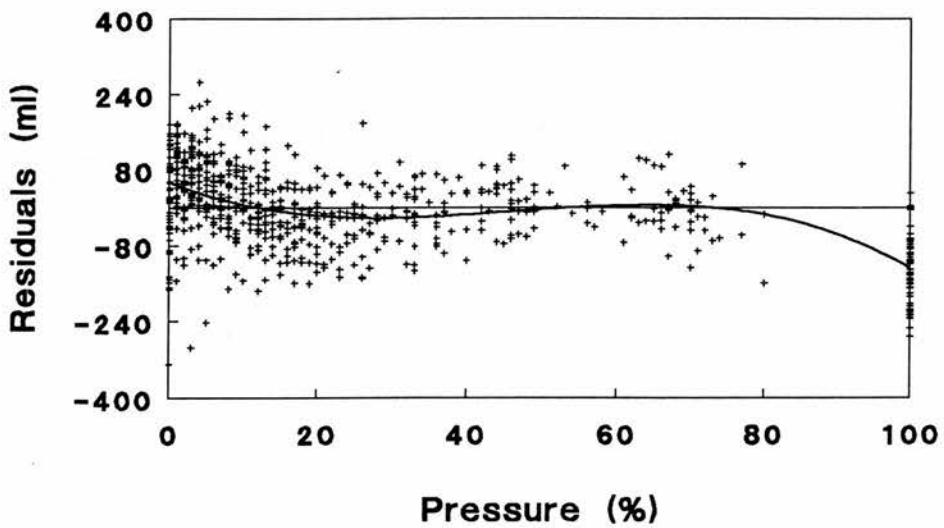
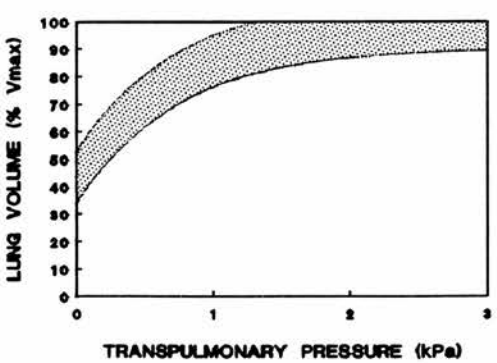


Figure 3.2. Plot of residuals for all fitted curves for 18 sheep. Pressures are expressed as percentages of the range between the minimum (0%) and maximum (100%) pressure recorded for each set of data points.
The fitted curve is of the form $Y = a+b\ x + c\ x^2 + d\ x^3$.

Figure 3.3. Relationship between transpulmonary pressure and lung volume (expressed as percentage of predicted Vmax) in normal sheep. The shaded area represents the 95% confidence interval.



A significant positive correlation was apparent between Cst and $V_{A,eff}$ (r_s = 0.505; P<0.025) whereas no significant relationship existed between K and $V_{A,eff}$ (r_s = 0.183; P>0.05) (Appendix 3.7).

3.4 DISCUSSION

The best-fit exponential curve was chosen by maximizing the coefficient of determination obtained by least-squares regression analysis of the linearised data. Visually, exponential curves appeared to closely fit the pressure-volume data and this was reflected in the regression analyses, where 96.11-99.74% of the variation in the dependent variable was explained by the fitted curve. Indeed, in only 4 cases did the unexplained variation exceed 2% of the total variation.

Systematic deviations from the exponential functions were frequently noted at high transpulmonary pressures and much of the error variance was attributable to this portion of the curve. The implications of these observations are that (a) in sheep, lung volumes measured at a transpulmonary pressure of 3kPa are not maximal, and (b) an exponential curve is not the most accurate description of the relationship between transpulmonary pressure and lung volume in this species. Examination of a previously published pressure-volume curve obtained from an anaesthetized sheep (Colebatch and Halmagyi, 1961) suggests that the first assumption might be valid. With regard to the second assumption, it is quite likely that a more complex mathematical formulation could be derived to better fit the pressure-volume data from sheep, just as in humans a hyperbolic-sigmoid model appears to offer a better fit to the full range of pressure-volume data than the exponential model (Murphy and Engel, 1978). However, the relative simplicity and lack of constants in the exponential equation together with the predictable effect of a change in any constant on the shape and position of the curve weigh heavily in its favour and, in the author's opinion, compensate for the poor description of the curve at high transpulmonary pressure. Certainly, it would appear that the exponential constant K holds several advantages over Cst measurements in describing pressure-volume curves. Firstly, the full range of data is used in the exponential analysis, whereas only data points in the linear portion of the pressure-volume curve are used in the calculation of Cst. Secondly, whereas Cst is positively correlated with $V_{A,eff}$, K is independent of this variable and thus is a more useful index of distensibility for use in comparative studies or when studying the effects of lung disease on pulmonary mechanics.

Data points at low volume levels tend not to conform to the exponential model in humans (Colebatch *et al.*, 1979a; Gibson *et al.*, 1979). Below about 46% TLC in seated healthy men (Sutherland *et al.*, 1968) there is an inflection in the curve caused primarily by the sequential closure of small airways and oesophageal artefacts. Inclusion of data points below this point of airway closure in the exponential curve fitting analysis leads to underestimation of *K* and overestimation of Vmax (Gibson *et al.*, 1979). Inflection points were not well defined in our ovine pressure-volume curves and curves appeared to underestimate Vmax rather than the converse. In addition, exclusion of data points below 50% TLC did not improve the quality of curve fit. It is therefore concluded that the factors responsible for curve inflection have less influence in the low volume range in anaesthetized sheep relative to humans and the whole data range from FRC to TLC can be used for exponential curve fitting.

The results of the pooled regression indicate that the ratio of A/Vmax% is approximately 57%. Individual values varied from 48.2 to 75.4% (median 61.2%). This ratio describes the position of the curve relative to the transpulmonary pressure axis and is considerably lower than the equivalent ratio previously described for humans (Colebatch *et al.*, 1979a; Gibson *et al.* 1979; Knudson and Kaltenborn, 1981) indicating that relative to these studies, the sheep pressure-volume curves are displaced to the left. Knudson and Kaltenborn (1981) reason that differences in oesophageal balloon volume can account for much of the variation in this ratio seen in human studies and comment that, compared to measurements of *K* which are seemingly less variable between studies, the value of this ratio to compare various studies is limited. Analysis of data from pressure-volume curves obtained from 6 sheep over a five-month period (Chapter 2) indicated the converse (Appendix 3.10), that the position of the curve relative to the transpulmonary pressure axis remained relatively constant (CV 12.91) whereas *K* was more variable over this period (CV 23.13).

K does not seem to be volume dependent in humans (Colebatch *et al.*, 1979a) although a progressive increase in this parameter occurs with age (Colebatch *et al.*, 1979b) such that between 20 and 80 years of age the value of *K* would be expected to increase from approximately 1.224 to 1.748 kPa. The age related increase in *K* is believed to be due to increased unstressed alveolar dimensions and reduced total surface acting forces (Colebatch and Ng, 1986). Although, no relationship between age and K could be demonstrated in sheep in the present study (Appendix 3.8) this probably reflects the weighted age distribution and relatively low numbers of the sample population rather than any intrinsic physiological difference in ageing sheep relative to humans. The relationship between airspace size and *K* has been demonstrated in excised human lungs (Greaves and Colebatch, 1980) and for lungs of several mammalian species (Haber *et al.*, 1983). Thus, species with very small alveoli would be expected to have relatively large surface acting forces and

low values of K. In this regard it is interesting that the values of K obtained in this study for adult sheep (median 1.456 kPa; range 1.091-2.119) are similar to those reported for humans; this despite sheep having smaller alveolar dimensions and correspondingly greater alveolar surface density relative to humans (Gehr *et al.*, 1978; Warner *et al.*, 1986). Whether this represents an intrinsic difference between species with regard to the type and extent of tissue related forces or a difference due to methodological inconsistencies between studies remains to be ascertained.

In conclusion, our studies indicate that an exponential equation adequately describes the whole pressure-volume curve of normal anaesthetized sheep. The index of distensibility K, is independent of lung volume and offers a means of comparing lung distensibility both with other species and during lung disease in this species.

3.5 SUMMARY

Static pressure-volume curves were generated from data obtained from 18 normal anaesthetized adult sheep. Lung volumes were determined by helium dilution. An exponential curve of the form $V = Vmax - A \cdot e^{-K \cdot P}$ was fitted to the pressure-volume data from each sheep where P is the static recoil pressure, Vmax represents the volume asymptote, A is the difference between Vmax and the intercept on the volume axis and K defines the slope and hence the shape of the P-V curve. Quality of fit of the data was assessed visually, by means of a sign test and a runs test and by the coefficient of determination (r^2). Exponential equations were found to adequately describe the shape of the pressure-volume curve in sheep. The exponent K was not correlated with effective alveolar volume ($V_{A,eff}$) ($r_s = 0.183$; $P>0.05$). Static lung compliance was determined over a volume range from FRC to FRC plus 400mls. Measurements of static lung compliance were significantly correlated with measurements of effective alveolar volume ($V_{A,eff}$) ($r_s = 0.505$; $P<0.025$). In the ovine, the exponent K, an index of distensibility, is independent of lung volume and offers a means of assessing lung distensibility in this species.

CHAPTER 4

LUNG MECHANICS, LUNG VOLUME, AND SINGLE-BREATH TRANSFER FACTOR FOR CARBON MONOXIDE IN SHEEP NATURALLY INFECTED WITH MAEDI-VISNA VIRUS

4.1 INTRODUCTION

The principal pathologic feature of maedi-visna virus (MVV) infection in sheep is a slowly progressive lymphoid interstitial pneumonia (Georgsson *et al.*, 1976) that causes dyspnoea, reduced exercise tolerance, and loss of body condition (Palsson, 1976). The MVV shares considerable sequence homology and morphological similarities with human immunodeficiency virus (HIV), also a lentivirus, the aetiological agent of the acquired immune deficiency syndrome (AIDS)(Gonda *et al.*, 1985). Lymphoid interstitial pneumonia (LIP) is a recognized pulmonary complication of AIDS in adults (Teirstein & Rosen, 1988) and children (Pitt, 1991), however, the pathogenesis of LIP in people has yet to be elucidated, as have appropriate diagnostic and therapeutic measures (Pitt, 1991). It is likely that the primary lymphoproliferative response seen in association with MVV-infection and with AIDS is a result of similar immune dysregulation (Lairmore *et al.*, 1986). As a consequence, further investigation into the pathogenesis of MVV-infection is warranted.

Functional changes in human interstitial lung disorders are characterised by reduction in compliance, lung volumes, and transfer factor for carbon monoxide (Wanner, 1980; Van Noord *et al.*, 1989). Physiological assessment of these functional changes, in combination with other investigative techniques, such as radiography, have proved useful in quantifying and monitoring the progression of LIP associated with AIDS in human beings (Morris *et al.*, 1987; Popa, 1988).

Clinical signs of MVV infection are apparent only in the later stages of the natural disease, and little is known about disease progression in the preclinical phase. Meaningful investigation of the pathogenesis of naturally acquired maedi-visna requires the use of minimally invasive techniques to stage and monitor the course of disease. The use of physiologic techniques to measure lung function of infected sheep is a logical progression from clinical examination, which tends to identify sheep only in the latter stages of the disease.

To the author's knowledge, lung functional changes associated with MVV infection have not been described; however, if present, these changes may be of value for staging the disease in the

preclinical phase. It was, therefore, considered appropriate to examine sheep with natural MVV infection to characterize the effect of this disease on static and gas exchange properties of the lungs.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Over a period of 19 months from 1/10/91 to 7/5/93 a total of 77 lung function measurement procedures were completed on 51 anaesthetized adult Texel ewes derived from a flock naturally infected with MVV (Watt *et al.*, 1992). Serological status was established with the agar gel immunodiffusion test (Winward *et al.*, 1979) and sheep classified as infected if associated with at least one previous positive gel test. Details of the naturally infected sheep used in this study are shown in appendix 4.1. The sheep had no evidence of respiratory tract disease unrelated to MVV infection as indicated by results of clinical examination and routine hematologic analysis.

In addition to routine subjective clinical appraisal, a subset of the sheep were examined in a standardized semi-quantitative manner using clinical worksheets that identified the clinical criteria adjudged to be of importance in the objective assessment of respiratory disease. Scores were assigned according to the presence or absence of these criteria and arbitrary weightings applied such that a clinically normal sheep would have a score of zero and the maximum score possible would be 370 (Appendix 4.2, 4.2.1 & 4.2.2).

4.2.2 Anaesthesia

Anaesthetic protocol was as described in chapter 2 (2.2.2).

4.2.3 Measurements

The precise methodology employed to measure static lung compliance (Cst), effective alveolar lung volume ($V_{A,eff,}$) single-breath transfer factor for carbon monoxide ($T_{L,CO,'sb'}$) and transfer factor corrected for lung volume ($T_{L/VA}$) has been described previously (Chapter 2; section 2.2.3). Where appropriate, exponential curve fitting analysis was carried out as described in chapter 3 (3.2.5).

4.2.4 Statistical analysis

Nonparametric statistical tests were employed. Three determinations of static lung compliance, lung volumes and transfer factor were made on each sheep, and the mean value was calculated. Values obtained were compared with predicted values for clinically normal seronegative sheep of the same breed and sex and of similar age using the appropriate equations relating lung function variables to bodyweight (Chapter 2, section 2.3, table 2.1).

Pooled data from all MVV-infected sheep were compared with predicted normal values using the Mann-Whitney U test. In order to assess whether deficits in lung function were age related, the sheep were grouped according to age into 5 groups (group 4 (3.5-4.5 years), group 5 (4.5-5.5 years), group 6 (5.5-6.5 years), group 7 (6.5-7.5) and group 8 (>7.5 years)). The Kruskal-Wallis one-way analysis of variance was used to assess whether the extent of lung function deficit was related to the age group of the sheep and linear regression analysis used to assess the association between age and lung function for the whole data set. For individual age groups the Mann-Whitney test was used to compare observed results with predicted.

In those sheep subjected to the clinical scoring scheme (Appendix 4.3) the relationship between lung function deficit and clinical score was assessed using the Spearman rank correlation coefficient.

Exponential curve fitting analysis was carried out on pressure-volume data from 12 MVV-infected sheep. This data was statistically compared to data collected from 11 control sheep using the Mann-Whitney test.

4.3 RESULTS

Results of Cst, $V_{A,eff}$, $T_{L,CO,'sb'}$ and $T_{L/VA}$ measurements made in 51 sheep naturally infected with MVV are given in appendix 4.2. Predicted values for seronegative sheep of equivalent bodyweight are also listed (Appendix 4.3).

$V_{A,eff}$, $T_{L,CO,'sb'}$ and $T_{L/VA}$ values were significantly less than predicted for the whole data set, whereas Cst was not significantly changed (Appendix 4.4).

Age related lung dysfunction is depicted graphically in figures 4.3.1-4.3.4. Sheep are grouped according to age (n = 4, 6, 31, 30 and 6 for groups 4 - 8 respectively). The graphs comprise

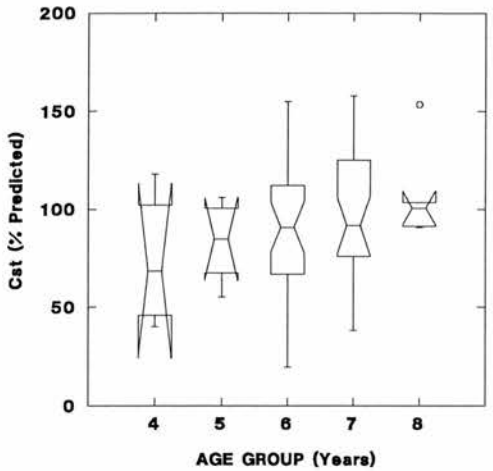


Figure 4.3.1 Cst values for MVV-infected sheep expressed as percent predicted value. There is no significant difference between observed and predicted values for any age group.

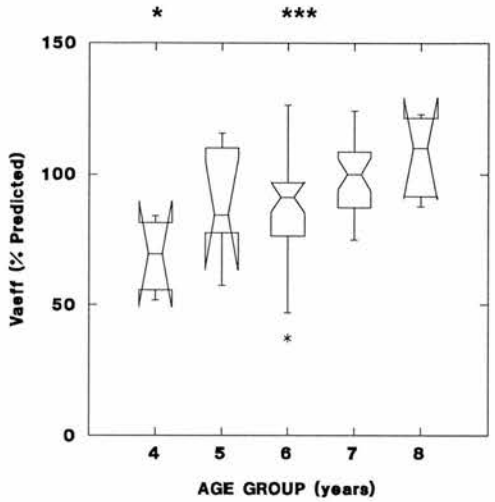


Figure 4.3.2 $V_{A,eff}$ values for MVV-infected sheep. Values for age groups 4 & 6 are significantly less than predicted ($P<0.05$ & $P<0.005$ respectively).

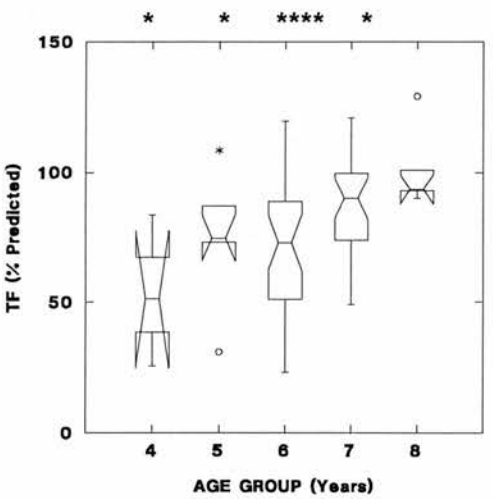


Figure 4.3.3 $T_{L,CO,'sb'}$ values expressed as percent predicted for MVV-infected sheep. All but the oldest group of sheep have values significantly less than predicted (* = $P<0.05$; **** = $P<0.001$).

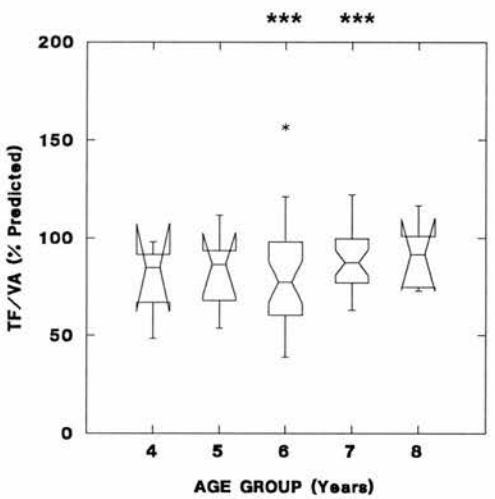


Figure 4.3.3 $T_{L/VA}$values expressed as percent predicted for MVV-infected sheep. Groups 6 & 7 had values significantly less than predicted. (*** = $P<0.005$).

boxplots in which the centre horizontal line depicts the median value i.e. the point at which the ordered batch of numbers is split in half. The upper and lower horizontal lines (hinges) split the remaining halves in half again, the distance between them representing the interquartile range. The vertical lines indicate the range of values falling within 1.5 interquartile ranges of the upper and

lower hinges. Values falling outside these ranges are plotted with asterisks (between 1.5 and 3 interquartile ranges from hinges) or open circles (over 3 interquartile ranges from hinges). The boxes are notched at the median values and return to the full width at the lower and upper confidence interval values. Boxplots were plotted using commercially available statistical software (Systat for Windows, v.5; Systat Inc., Evanston, IL, USA). Legends above each plot indicate whether groups differ significantly from predicted values using the Mann-Whitney test (* = $P<0.05$, ** = $P<0.01$, *** = $P<0.005$, **** = $p<0.001$). Results of the Mann-Whitney tests are given in appendix 4.4.

The Kruskal-Wallis one-way analysis of variance by ranks was used to assess whether the age groups differed significantly between each other i.e. whether the different age groups were from different populations. The results indicated that significant age related differences in $V_{A,eff}$ and $T_{L,CO,'sb'}$ occurred within the MVV-infected sheep (Appendix 4.5). The relationships between age and percent predicted lung function for the ungrouped data sets are shown in appendix 4.6.1-4.6.4, this relationship being significant for Cst (r = 0.278;$P<0.05$), $V_{A,eff}$ (r = 0.491;$P<0.001$) and $T_{L,CO,'sb'}$ (r = .0.476;$P<0.001$).

Spearman correlation coefficients indicated significant negative correlations between clinical score and percent predicted $V_{A,eff}$ and $T_{L,CO,'sb'}$ (r_s = -0.358; $P<0.05$ & r_s = -0.389; $P<0.05$ respectively). There was no significant correlation between clinical score and percent predicted Cst or $T_{L/VA}$ (r_s = -0.225 & r_s = -0.199 respectively).

In the comparison of exponential constants and coefficients from curve analysis of MVV-infected and control sheep pressure-volume curves (Appendix 4.7) there was a significant reduction in Vmax and the ratio of A/Vmax as indicated by Mann-Whitney U tests ($P<0.05$ for both tests)(Appendix 4.8).

4.4 DISCUSSION

Clinical examination and routine haematology were used to determine that intercurrent respiratory disease unrelated to MVV infection did not feature in the examined cases. In addition, stringent anthelminthic dosing protocols were adopted prior to the study such that lungworm infection would be a negligible risk. Concurrent bronchoalveolar lavage studies (Luján *et al.*, 1993) and pathological monitoring of the flock (Watt *et al.*, 1992) also served to illustrate the respiratory status of both individual animals and the flock as a whole. However, in the absence of a 'Gold standard' window on the respiratory tract such as lung biopsy the limitations of clinical or laboratory

techniques as methods of detecting subclinical respiratory disease should be understood and results interpreted accordingly.

The normal pulmonary interstitium is composed of connective tissue components (collagen, elastic fibres, proteoglycans, and fibronectin), mesenchymal cells, and inflammatory and immune effector cells (Crystal *et al.*, 1981). The most obvious physical effect of LIP is a thickening of the interstitial space with consequent alteration of the number, form, and location of its cellular and noncellular elements. The anticipated physiologic consequences of such structural alterations include changes in compliance, lung volumes and transfer factor. Because the pressure-volume characteristics of the lung are largely determined by the tissue network of collagen and elastic fibres (ie, the noncellular elements in the interstitium)(Mead, 1961), any change to this network is liable to lead to changes in compliance. Static lung volumes are influenced by lung and thoracic wall recoil pressures (Cotes, 1979) and the volume of tissue and fluid within the thoracic cavity (Ries, 1989). Changes in both determinants would be anticipated in LIP.

Defined as the rate of transfer of a gas per unit driving pressure between the alveoli and the erythrocytes within the pulmonary capillaries, transfer factor is affected by a number of structural lung dimensions, including lung volume, the path length for diffusion in the gas phase, the surface area of the alveolar capillary membrane, and the volume of blood in the capillaries perfusing ventilated alveoli (Cotes, 1983). Changes to several of these dimensions occur in LIP; thus, alterations in transfer factor would be anticipated.

In advanced maedi, the lungs are heavy and firm and tend not to collapse when removed from the thorax (Georgsson & Pálsson, 1971). These observations might suggest that affected lungs are less compliant than normal, however although a trend towards reduced Cst was apparent for the whole data set, no significant difference between observed and predicted Cst was noted. Changes in compliance in people with interstitial lung disease seem to reflect predominantly fibrosis (Keogh & Crystal, 1980) and are not correlated with inflammatory events (Fulmer *et al.*, 1979). On this basis, since fibrosis is on the whole inconspicuous in maedi (Georgsson *et al.*, 1976), a reduction in compliance is presumably not a necessary consequence of pathology.

Vmax was less and the ratio of A/Vmax significantly greater for MVV-infected sheep when compared with controls whereas the index of distensibility (K) was not significantly altered. Figure 4.4 demonstrates the exponential curves obtained from the composite MVV-infected and control data with volume expressed as percent Vmax (constants and coefficients were obtained by simply

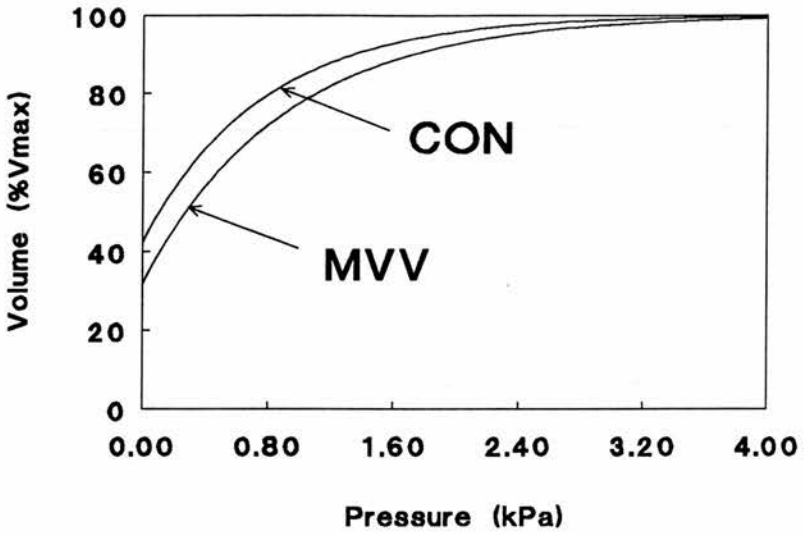


Figure 4.4 Exponential curves fitted to composite pressure-volume data from MVV-infected and control sheep.

averaging the grouped data). The equations describing these curves are Vol (%Vmax) = 100 - $68.3 \cdot e^{-1.121 \cdot P}$ for the MVV-infected sheep, and Vol (%Vmax) = 100 - $57.7 \cdot e^{-1.315 \cdot P}$ for the control sheep. These results indicate that compared to control curves, pressure-volume curves from MVV-infected sheep are displaced to the right i.e. for a given lung volume (expressed as percent Vmax) there is an increase in elastic recoil. Crawford *et al.* (1987) demonstrated that in human subjects given intravenous atropine, pressure-volume curves undergo a parallel shift to the left with no change in *K*. They attributed this loss of lung elastic recoil after intravenous atropine to the abolition of vagally mediated peripheral bronchomotor tone and suggested that in the normal subject, bronchomotor tone contributes to the elastic recoil of the lung. It is tempting to speculate that, in maedi-visna, the distinct smooth muscle hyperplasia seen in the lung periphery and especially at the level of the alveolar ducts is involved in the increased elastic recoil. Although the exact pathophysiological role of the smooth muscle hyperplasia is unknown, one theory is that it may serve to compensate for the lack of elastic recoil resulting from the thickening of the interalveolar septa and fragmentation and destruction of elastic fibres seen in the disease (Georgsson *et al.*, 1976). Accordingly, lung elastic recoil in MVV-infected animals may presumably be influenced by the quantity and functional tone of the smooth muscle in the lungs.

$V_{A,eff}$ was significantly reduced in the MVV-infected sheep. Similar changes in lung volumes are recognized in human beings with idiopathic pulmonary fibrosis (West, 1987) and sarcoidosis (Crystal *et al.*, 1984) and may also be seen in people with LIP (Kradin & Mark, 1983;

Popa, 1988). Such changes are presumably a consequence of the tissue filling effect of the interstitial reaction but altered lung elastic recoil may also contribute.

$T_{L,CO,'sb'}$ and $T_{L/VA}$ values were significantly reduced in the MVV-infected sheep. This is a common finding in human beings with interstitial lung disease (Crystal *et al.*, 1981) in whom transfer factor measurements may be useful for the follow-up evaluation of individual cases of sarcoidosis and interstitial fibrosis (Crapo & Forster, 1989). The $V_{A,eff}$ value is the single greatest source of variability of $T_{L,CO,'sb'}$ (Ferris, 1978) with a decrease in $V_{A,eff}$ exposing a smaller alveolar membrane surface area to participate in gas exchange. The significantly lower $V_{A,eff}$ in MVV-infected sheep may, therefore, contribute to the observed difference in $T_{L,CO,'sb'}$. That other factors are involved in the reduction of the transfer factor in MVV-infected sheep is evidenced by the still significant reduction in transfer factor after correction for lung volume. Such factors are likely to include alterations in ventilation-to-perfusion ratio, pulmonary hypoperfusion, capillary transit time and thickening and functional alterations of the interalveolar membrane brought about by the cellular infiltration (Nunn, 1977).

These physiologic changes, specifically reduction in lung volumes and transfer factor, are consistent with those found in people with interstitial lung disease (Wanner , 1980; Van Noord *et al.*, 1989). Knowledge that measurable changes in lung function do occur in natural MVV infection provides a basis for further investigation of the potential of lung function studies as a means of staging this disease.

Clinical score was negatively correlated with percent predicted $V_{A,eff}$ and $T_{L,CO,'sb'}$. The correlations were weak however, indicating the deficiencies of clinical assessment in quantifying subclinical respiratory disease.

Cst, $V_{A,eff}$ and $T_{L,CO,'sb'}$ expressed as percent predicted values were positively correlated with age. This was a surprising finding as it is generally assumed that, being a chronically progressive lung disease, older sheep would have comparatively more severe lung dysfunction. One justified criticism might be aimed at the fact that group sizes were unequal and that the age distribution of lung dysfunction might represent statistical artefact. However, direct comparison of age groups 6 & 7 (which are the largest groups and of a comparable size) using the Mann-Whitney U test demonstrates that the younger sheep have significantly lower $V_{A,eff}$ and $T_{L,CO,'sb'}$ values ($P<0.01$ & $P<0.005$ respectively). The history of the flock from which these MVV-infected sheep were derived has been described (Watt *et al.*, 1992). The sheep in groups 7 & 6 were born between 1985 and 1987. At this time the flock was still being managed by the original owner and was

experiencing an increasing seroprevalence of MVV (8% in December 1985 - 13% in June 1986), until in 1988, at the time the youngest age group in the present study (group 4) would be born, 64.7% of the pedigree sheep were seropositive (Watt *et al.*, 1992). Epidemiological observations would therefore suggest that the risk of infection, either by lactogenic or horizontal transmission, would have been greater for sheep of the younger age groups when they were lambs. Pregnancy rates, nutritional or managemental factors and alterations in the pneumovirulence of viral strains are some of the alternative possibilities that may have contributed to the age related differences in pulmonary dysfunction seen.

Taken together the results indicate that MVV-infection is associated with variable lung dysfunction and suggest the potential value of these measurements in assessing subclinical lung disease. However, this study was simply a functional assessment in which clinical criteria were largely relied upon to limit the chances of sheep with intercurrent respiratory disease unrelated to maedi appearing within the data set. However, as the poor correlations between clinical score and lung function variables suggest, the reliability of clinical examination is questionable and it is possible that sheep with intercurrent respiratory disease unrelated to maedi did appear within the data set. To exclude this possibility would require the use of antemortem procedures such as lung biopsy or radiography (with their own inherent limitations) or the follow-up of individual sheep at post mortem examination.

4.5 SUMMARY

Static lung compliance, static lung volumes, and transfer factor for carbon monoxide were measured in 51 anaesthetized adult Texel ewes derived from a flock naturally infected with MVV. Values obtained were compared with predicted values for clinically normal seronegative sheep of the same breed and sex and of similar age using the appropriate equations relating lung function variables to bodyweight (Chapter 2, section 2.3, table 2.1). In addition exponential curve fitting analysis was carried out on pressure-volume data from 12 MVV-infected sheep. This data was statistically compared to data collected from 11 control sheep.

In MVV-infected sheep $V_{A,eff}$, $T_{L,CO,'sb'}$ and $T_{L/VA}$ values were significantly less than predicted. The results of exponential curve fitting analysis indicated that Vmax was less and the ratio A/Vmax significantly greater in MVV-infected sheep. These results indicate that MVV-infected sheep have reduced lung volumes and gas diffusing capabilities and have increased lung elastic recoil when compared to control sheep. Percent predicted $V_{A,eff}$ and $T_{L,CO,'sb'}$ values were weakly negatively correlated with clinical score.

Age related changes in lung dysfunction in MVV-infected sheep indicated an overall improvement in lung function in the older sheep in the flock. This finding may be explained by the epidemiological history of seroprevalence at the time of birth of respective age groups i.e. younger age groups being exposed to a greater natural challenge than older groups.

CHAPTER 5

MORPHOMETRIC ANALYSIS OF SHEEP LUNGS

5.1 INTRODUCTION

MVV-infected sheep have reduced lung volumes and gas diffusing capabilities and have increased lung elastic recoil when compared to control sheep (Chapter 4). It is not known how these changes relate to lung pathology. However, this question is of vital importance if lung function analysis has a role to play in staging maedi in the preclinical phase i.e. in a predictive capacity. Prior to interpreting lung function in relation to pathology in maedi it is important to define the normal structure of the sheep lung. In this regard it is necessary to adopt a disciplined approach to the preparation and sampling of lung tissue specimens for histological evaluation, and measurement techniques applied should be of proven accuracy and provide data of relevance to functional measurements. This structural evaluation of lung anatomy is an application of the science of morphometry, defined as a body of methods for obtaining numerical information about anatomical structure, macroscopic or microscopic, in terms of such quantities as volume, surface area and size and number of components (Aherne & Dunnill, 1982)

It is prudent to subjectively define the portion or compartment of the lung that is involved in the pathological process such that structural analysis can be efficiently directed towards an area likely to be involved in functional disturbance. Accordingly, as the predominant pathological change involves the pulmonary parenchyma in maedi, and there is negligible evidence for significant pathology involving the airways or blood vessels (Georgsson & Pálsson, 1971), structural analysis should be directed towards this lung compartment. The following describes the preparation of ovine lungs for morphometric evaluation, the measuring techniques used in evaluation and the results of structural analysis of the pulmonary parenchyma in sheep lungs.

5.2 MATERIALS AND METHODS

5.2.1 Source of material

The left lungs from 10 female sheep of mixed breed were used for morphometric evaluation. Macroscopic studies were completed on 7 lungs and microscopic studies in the remaining 3. Details of sheep used to provide material for evaluation are given in appendix 5.1.

5.2.2 Collection of material

Sheep were euthanased by intravenous injection of pentobarbitone sodium BP (Lethobarb, Solvay-Duphar Veterinary, Southampton, UK) and subsequently exsanguinated. The lungs and mediastinal contents were removed from the thorax, with particular care taken to avoid damaging the pleural surface in any way.

The lungs were grossly examined and subjective findings noted and recorded by an experienced respiratory pathologist (NJW). The heart and mediastinal contents including mediastinal lymph nodes were carefully trimmed from the lungs. The total lung weight (TLW) and left lung weight (LLW) were measured and recorded.

5.2.3 Tissue fixation

Lung tissue was fixed by airway instillation of paraformaldehyde. After clamping the lobar bronchi to the right lung, this lung was removed and the tracheal stump connected to the left lung was cannulated and connected to a reservoir of phosphate-buffered 4% paraformaldehyde (pH 7.2-7.4)(Appendix 5.2), at a head pressure of 2.5 to 3.0 kPa. This fixative was allowed to flow until the 'natural contours' of the lung were established (Figure 5.1). The left lungs were then floated in a tank

Figure 5.1 Left lungs after airway instillation of fixative to establish the 'Natural contours'. The lungs were then attached to a reservoir of fixative kept at a constant hydrostatic pressure of 2.5-3.0 kPa for a period of 4 days.

of the same fixative and inflation-fixed at a constant pressure of 2.5 to 3.0 kPa for a period of 4 days. The apparatus used is illustrated in appendix 5.3.

5.2.4 Macroscopic evaluation of fixed tissue specimens

Following fixation, the volume of the left lung was measured by water displacement using a plastic reservoir fitted with a discharge siphon (Aherne & Dunnill, 1982). Five repetitions of this volume measurement were completed to yield an acceptable mean value and standard error (Aherne & Dunnill, 1982). The total fixed lung volume (TLV_F) was inferred from the ratio of left lung weight to total lung weight.

The lung was then placed on a cutting board and the lung sectioned into 1cm thick transverse slices using an exquisitely sharp brain knife.

Pulmonary parenchyma, identified as the phase of interest for microscopic evaluation, is defined as that compartment of the lung with no structures (airways or blood vessels) with diameters greater than 1mm present. The rejected compartment, 'coarse non-parenchyma' (Weibel, 1984) will include all airways and blood vessels visible at a macroscopic level. Estimation of the volume fraction of parenchyma in the lung (V_{vp}) was achieved by direct point counting of the fixed transverse lung slices as described by Dunnill (1962). The transverse lung slices were laid out on the cuttting board and a transparent 300-point triangular pattern counting grid (distance between points, d = 1 cm) was overlaid each slice. The grid was examined and the type of tissue underlying each point was assessed and recorded as either parenchyma or non-parenchyma. The mean value for V_{vp} was calculated as n/N, where n is the total number of points falling on the parenchymal compartment and N is the total number of points falling on lung tissue *per se* for all the lung slices. The mean value of V_{vp} for the 7 lungs was calculated.

5.2.5 Tissue preparation for microscopic evaluation

Six slices were selected for tissue sampling from the region just caudal to the cranial margin of the ventral basal segment of the diaphragmatic lobe (Hare, 1955)(Appendix 5.4). The principle of stratified random sampling was applied to the selection of tissue blocks for morphometric evaluation (Dunnill, 1962). Using a transparent numbered grid overlay (Appendix 5.5) and computer generated random numbers 12 sites were selected for sampling tissue blocks from the six slices. Pins were placed through perforations at grid intersections to demarcate sites for trimming tissue blocks from the slices. Tissue blocks were not accepted if the selected area overlay

large conducting airways or blood vessels. Tissue blocks were trimmed to 1.9 x 1.9 cm using a suitable template.

Trimmed tissue blocks were placed in tissue cassette cases and embedded in paraffin. Sections were cut from each block at 7μm and stained with haematoxylin and eosin using standard histological techniques (Diagnostic Pathology Laboratory, Department of Veterinary Pathology, R.(D).S.V.S).

5.2.6 Microscopic evaluation techniques

12 sections were available for morphometric evaluation at the microscopic level. A semi-systematic selection procedure was used, in the following manner, to select fields within sections for morphometric analysis. The leading edge of each section was measured with the stage micrometer and the length (l) determined in millimeter divisions. An integer random number (n) was chosen between 1 and l, and the stage advanced to this position. If the examined fields satisfied defining criteria for parenchyma (absence of airways or vessels over 1mm diameter) morphometric measurements were made. Following measurements, or if fields failed to satisfy acceptance criteria, the stage micrometer was advanced by n divisions and assessment repeated. When the edge of the slide was reached, the opposite micrometer scale was altered by 1 division towards the edge opposing the leading edge of the section and the direction of scanning continued in the opposite sense.

Morphometric measurements were performed at a magnification of 400x using a multipurpose test grid (Weibel GW2: Graticules Ltd.) composed of a combined line and point system (Weibel *et al.*, 1966). All morphometric analyses were completed by the same operator. The volume densities of tissue (V_{vt}) and air (V_{va}) were calculated by relating the number of points landing on tissue to the total number of points counted, and the surface density (S_{vt}) was calculated by relating the number of line intersections with alveolar septae to the number of tissue point 'hits' (Freere & Weibel, 1966)(Appendix 5.6). Tissue sections were randomly selected from the pool of twelve sections for each sheep and six fields from each section were randomly selected and analysed as described above (after analysis each section was returned to the pool and could be randomly selected again). A total of 72 fields were therefore examined, giving a total point count of 3024 points per animal. Values were multiplied by the total volume of the parenchymal region (section 5.2.4) to calculate absolute volumes and surface areas as follows

Volume of lung parenchyma	TV_p	=	$TLV_F \cdot V_{vp}$
Total parenchymal tissue volume	$TV_{p,t}$	=	$TV_p \cdot V_{vt}$
Total parenchymal airspace volume	$TV_{p,a}$	=	$TV_p \cdot V_{va}$
Total airspace surface area of lung parenchyma	ASA_p	=	$TV_p \cdot S_{vt}$

Since all lungs were treated in an identical manner, it was considered unnecessary to calculate fixation or processing constants.

5.3 STATISTICS

To ensure that an adequate number of points were counted for both the macroscopic and microscopic volume estimations the relative standard error (RSE) i.e. the size of the standard error relative to the mean value of the count, was reduced to less than 5% (Aherne & Dunnill, 1982). An estimate of the number of points (n) necessary to reduce the RSE to within these limits was calculated from

$$RSE = \sqrt{1-V_V} / \sqrt{n}$$

where V_V is the volume fraction of the component under study (Aherne & Dunnill, 1982).

5.4 RESULTS

The constant-pressure inflation fixation procedure proved satisfactory in that lung tissue was adequately fixed after 4 days and uniform inflation was apparent throughout the lung.

Results of macroscopic evaluation of the fresh and fixed lung specimens are given in table 5.4.1. Details of macroscopic point counting measurements made are given in appendix 5.7 and summary results are given in table 5.4.2. Details of microscopic point counting measurements are given in appendix 5.8 and summary results and calculations are given in table 5.4.3.

Sheep No.	LLW	TLW	TLV_F	Subjective Pathology
	(kg)	(kg)	(ml)	
CON101	0.36	0.53	2323	NSF
CON103	0.35	0.53	2144	NSF
CON105	0.36	0.65	2326	NSF
AHGR59	0.67	1.70	3029	Classical maedi
AHGR36	0.60	1.45	2948	Classical maedi
AHBL29	0.20	0.60	2022	NSF
AHBL28	0.45	1.10	2346	Classical maedi. Some M.capillaris lesions
AHBL97	0.37	0.85	1728	Early maedi lesions
AHRD25	0.25	0.65	2197	NSF
CON032	0.22	0.55	2082	NSF

Table 5.4.1. Results of macroscopic evaluation of fresh and fixed lung specimens. LLW - left lung weight, TLW - total lung weight, TLV_F - total fixed lung volume, NSF - no specific findings i.e. macroscopically normal.

No. of lungs examined	V_{vp} (%)			
n	Average	S.D.	Median	Range
7	85.2	2.48	84.0	83.0 - 89.9

Table 5.4.2. Summary results of macroscopic point counting applied to transverse lung slices to estimate the volume of pulmonary parenchyma (V_{vp}).

			Sheep No.		
Variable	Unit	Calculation	CON101	CON103	CON105
V_{vt}	%	P_t/P_T	19.0	17.9	24.1
S_{vt}	cm^2/cm^3	$2 \cdot N_t/L_T$	592.0	630.8	715.8
s_t/v_t	cm^2/cm^3	$4 \cdot N_t/Z \cdot P_t$	3113.4	3532.5	2969.1

Table 5.4.3 Summary results of microscopic point and line intersection counting in 3 normal lungs. V_{vt} - tissue volume fraction, S_{vt} - surface density, s_t/v_t - surface to volume ratio for tissue, P_t - total number of point intersections, P_T - total number of points counted (72 fields x 42 points = 3024), N_t - total number of line intersections, Z - length of test line segment (at a magnification of x400, Z = 45μ), L_T - total length of test line (72 fields x 21 lines x 45 μ = 6.804 cm).

5.5 DISCUSSION

The three principal methods of lung inflation and fixation are the negative pressure formalin steam method of Weibel & Vidone (1961), the constant-pressure airway instillation of fixative described by Heard (1958)(a modification of which is used in this study), and the technique recommended by the American Thoracic Society (ATS)(Krahl *et al.*, 1959) which involves inflating the lungs by airway instillation until their 'natural contours' are established and then floating them in fixative.

Weibel (1963) describes the latter processes of lung fixation by airway instillation as being less than ideal in that fluid is substituted for air in the airways thereby obliterating surface tension forces vital for maintaining lung tissue architecture in the living animal, and leading to tissue distortions due to the weight of the heavy fluid mass. By fixing specimens in the inflated state using formalin steam vapour, the method of Weibel & Vidone (1961) appears to satisfy the limitations of the aforementioned techniques. However, this technique itself has limitations in that it is technically demanding and has received criticism regarding inadequate fixation, loss of gas through perforations in the lungs and uneven fixation as a result of vapour condensing and draining to dependent lung regions to give wet fixation (Wright *et al.*, 1974).

Given the technical demands and criticisms of the formalin steam method the choice of fixation procedure for the present study was limited to either the Heard (1958) or ATS (Krahl *et al.*, 1959) techniques. One advantage of the ATS technique is that it is easier to apply to lobectomies and pneumonectomies than the Heard technique which requires the placement of long term cannulae (McLean, 1988). However, concerns borne of subjective observations on a limited number of lungs inflated by the ATS technique prior to the commencement of this study, were that some degree of lung collapse occurred during the relatively slow fixation process. Indeed Heard (1958) developed the constant pressure technique out of concern that, unless kept under constant pressure, the lung will flatten and shrink as fixative leaks away through the pulmonary vessels. Given these concerns, the fact that equipment was available to provide constant pressure inflation fixation, and the fact that only whole lung specimens were to be fixed, the technique of constant pressure inflation as originally described by Heard (1958) was chosen for this study.

Most body tissues shrink during both fixation and histological processing and constants can be calculated to correct for this artefact if interstudy comparisons are of a primary concern. Where all material is treated in an identical fashion, as in this study, such corrections are largely irrelevant (Aherne & Dunnill, 1982). It is however of interest to compare the degree of lung tissue shrinkage

during fixation found in this study with previously published results. Lum & Mitzner (1985) compared the effects of 10% formalin fixation on fixed lung volume using pre-fixation measurements of TLC as a within subject basis for comparison. They studied several species and calculated a value of 0.83 for the fixed to physiological lung volume ratio measured in 3 sheep. Given that *in vivo* functional measurements of static lung volumes were available for 3 sheep included within the present study the broadly equivalent ratio of total fixed lung volume (TLV_F) to effective alveolar volume ($V_{A,eff}$) could be calculated.

Sheep No.	TLV_F ml	$V_{A,eff}$ ml	$TLV_F/V_{A,eff}$ ratio
CON101	2323	3937	0.59
CON103	2144	4418	0.49
CON105	2326	4299	0.54

It can be appreciated that these estimates for the $TLV_F/V_{A,eff}$ ratio are all considerably lower than that quoted by Lum & Mitzner (1985). Ratios for other species varied from 0.59 for the dog to 2.01 for the mouse (Lum & Mitzner, 1985). These authors discuss the possible reasons for interspecies variability under standard conditions of measurement and comment on the possible influence of alveolar size, the quantity of collagen and elastin in the lung and the fixative flow rate.

Lum & Mitzner (1985) suggest that differences in fixative and/or flow rate and methods used in determining TLC pre-fixation may increase interstudy variability in the fixed to physiological lung volume ratio. The known areas of difference between the current study and that of Lum & Mitzner (1985) concern the type of fixative and the method of TLC determination (an open chest preparation being used in the latter instance). Differences between formalin and paraformaldehyde in terms of fixation shrinkage are unlikely to account for a large part of the variation in fixed to physiological lung volume ratios. Lum & Mitzner (1985) obtained values for TLC of 1990 ± 200 mls for 5 sheep of bodyweight 40 ± 1.85 kg (mean ± standard error). These values are considerably lower than would be predicted for sheep of similar bodyweight both on the basis of the regression equations given in chapter 2 (table 2.1)(respecting the fact that a bodyweight of 40 kgs lies just below the defined regression range) for adult Texel sheep (approximately 3814 mls) and on the basis of the power law formulae of Stahl (1967) (approximately 2670 mls). It is therefore possible that either methodological differences (in the method of calculating TLC), or breed differences may be accounting for a large part of the observed discrepancy in fixed to physiological lung volume ratios between the present study and that of Lum & Mitzner (1985).

A further possibility is that differences may have existed between the studies with respect to the time between euthanasia and lung fixation. Lum & Mitzner (1985) comment that excised sheep lungs are difficult to inflate and suggest that post-mortem bronchoconstriction, as occurs in the guinea-pig (Lai *et al.*, 1984), may contribute to this phenomenom. Although no mention is made of timing in the study by Lum & Mitzner (1985), in the present study sheep lungs were inflated with fixative within 20 minutes of euthanasia. If smooth muscle is actively contracting during this time it is possible that it becomes fixed in a partially contracted state and therefore limits TLV_F. Further studies are required to elucidate whether there is any relationship between the time interval betwixt euthanasia and lung inflation, and the ratio of $TLV_F/V_{A,eff}$.

The principle of stratified random sampling was adhered to in the selection of tissue blocks for histological evaluation. The procedure described shows minor modifications to that described by Dunnill (1962) and Weibel (1963). By the process of systematically slicing the lungs into 1cm thick slices and applying random sampling procedures to these it was theoretically possible to sample any block of lung parenchyma within the sampled portion. The premise behind this procedure is that the sample obtained is truly representative of the whole lung. This will only be the case if the structure of lung parenchyma is truly random, and in the normal lung this is considered to be so (Aherne & Dunnill, 1982). The stratified random sampling procedure was extended to the selection of fields within sections, whereby the first field was selected on the basis of a random number and the remaining fields were kept a constant distance apart (again determined by the random number).

The volume of lung parenchyma (V_{vp}) varied between 83.0 and 89.9% of TLV. There was no significant difference between those lungs with macroscopic evidence of maedi lesions (n=4) and those with no specific gross abnormalities (n=3) as assessed using the Mann-Whitney U test (P=0.593). The mean value for V_{vp} of 85.2% compares with 90% for human lungs fixed using formalin steam vapour under negative pressure (Weibel, 1963).

Results of microscopic morphometric evaluation of lung parenchyma from the present study are compared with previous studies in the sheep and human in table 5.5.1. The results of the present study appear to be in good agreement with those of Warner *et al.* (1986), however this agreement may in part be fortuitous as the optical resolution at which analyses were carried out differed considerably between the respective studies. It is known that S_{vt} increases approximately with the 0.1 power of magnification, (Gehr *et al.*, 1976,1978) thus the observed agreement between studies for S_{vt} is unexpected. Glutaraldehyde, which is associated with minimal shrinkage (Aherne & Dunnill, 1982), was used as the fixative in the study by Warner *et al.* (1986) whereas

paraformaldehyde, which is associated with shrinkage, was used as the fixative in the present study. Thus differences in

Variable	Unit	Present study	Warner *et al.* (1986)	Weibel (1963)	Weibel (1963)	Gehr *et al.* (1978)
Species	-	Ovine	Ovine	Human	Human	Human
n	-	3	4	5	5	8
BW	kg	81	42	-	-	74
TLV_F	ml	2264	1915	1958	1958	4341
V_{vt}	%	20	19	-	-	14
S_{vt}	cm^2/cm^3	646	660	201[1]	149[2]	371
ASA_p	m^2	124	103	35[1]	64[2]	143
Magnification	-	x400	x8,500	L.M.	L.M.	x11,200

Table 5.5.1 Morphometric results of the present study compared to previous studies in the ovine and human species. The total airspace surface area of lung parenchyma (ASA_p) was calculated from $TV_p \cdot S_{vt}$ assuming that $V_{vp} = 0.85$. [1,2] calculated from the authors own data, figures are for [1] processed and [2] fresh lung.

tissue shrinkage could contribute towards cancelling out the effect of increased optical resolution on the measurement of S_{vt}.

Differences in methodology can largely preclude valid interspecific comparisons, however, if respective electron and light microscopic studies are compared, there does appear to be obvious differences between ovine and human lung morphometry in that, as noted by Warner *et al.* (1986), S_{vt} is comparatively much greater for sheep than for humans i.e. alveoli are smaller in sheep. V_{vt} is also greater for sheep lungs than for human.

5.6 SUMMARY

The left lungs from 10 sheep were prepared for macroscopic (n=7) and microscopic (n=3) morphometric evaluation. Inflation fixation using 4% paraformaldehyde fixative at a constant-pressure of 2.5-3.0 kPa for a period of 4 days adequately fixed lung tissue and provided uniform lung inflation. The left lung was sliced into 1cm thick transverse slices. The volume fraction of the fixed lung volume occupied by pulmonary parenchyma (V_{vp}) was estimated by the process of point counting to be 0.85. V_{vp} did not differ between lungs with macroscopic evidence of maedi and macroscopically normal lungs (P = 0.593). The ratio of fixed to physiological lung volume ($TLV_F/V_{A,eff}$) ranged from 0.49 to 0.59 in 3 normal lungs. A stratified random sampling procedure

was used to sample 6 lung slices from the mid-diaphragmatic lobe of 3 normal lungs for microscopic morphometric evaluation. Point and line intersection counting were executed on randomly selected fields using a suitable light microscope graticule at a magnification of x400. Values for tissue volume fraction within the lung parenchyma (V_{vt}) ranged from 0.18 to 0.25 and values for alveolar surface density (S_{vt}) ranged from 592 to 716 cm^2/cm^3 . These results indicate that relative to human lungs, sheep lung parenchyma has a larger tissue volume fraction and a larger surface area per unit volume i.e. smaller alveoli.

CHAPTER 6

QUANTITATIVE LUNG MORPHOMETRY AND THE RATIO OF FIXED TO PHYSIOLOGICAL LUNG VOLUME

6.1 INTRODUCTION

It is often desirable to interpret lung function in terms of the physical properties of this organ and to relate these properties to associated lung structure at the light and electron microscope levels. However, on opening the thorax, the lungs collapse to a degree that depends on these physical properties, leading to considerable variation in the 'tissue state' after fixation, and consequently limiting the analysis of structure-function correlations. Accordingly, various methods have been adopted to control the level of lung inflation during fixation such that morphometric data can be related both to functional data within the same animal and with other studies in the same or different species. The underlying rationale is to reproduce in the fixed lung a consistent degree of inflation from which the lung structure can be related to the corresponding physiological state under similar conditions. However, studies of the most popular method of inflation fixation, that of airway instillation of fixative using a constant infusion pressure, have demonstrated a large interspecies variation in the ratio of fixed to physiological lung volume (Lum & Mitzner, 1985). This implies that, for quantitative interspecies physiologic studies, variable lung shrinkage caused by fixation must be recognised and measured (Lum & Mitzner, 1985). Within species, the fixed lung volume achieved following airway instillation is affected by the type of fixative, the fixative flow rate and infusion pressure (Hayatdavoudi *et al.*, 1980) and may also be influenced by the elastin content of parenchyma and pleura within the lung (Sobin *et al.*, 1982). Previous observations suggested that, in sheep, the ratio of fixed to physiological lung volume is variable (Chapter 5) and subjective opinion was that the delay between euthanasia and lung inflation affected this ratio. Accordingly this study sought to investigate, in control sheep, the relationship between the delay betwixt euthanasia and lung inflation and the fixed to physiological lung volume ratio.

6.2 MATERIALS AND METHODS

6.2.1 Animals

13 sheep (bodyweight 40-82 kgs) of mixed breed were used in this study. Details of sheep used are given in appendix 6.1. All were managed under identical conditions prior to the study.

6.2.2 Lung volume measurements

Immediately prior to euthanasia, the sheep were anaesthetized and physiologic measurements of lung volume were made. The protocol for anaesthesia and lung volume measurements was identical to that previously described (Chapter 2; sections 2.2.2-3). 3 determinations of effective alveolar volume ($V_{A,eff}$) were completed for each sheep.

6.2.3 Lung fixation

Immediately following lung volume determinations the sheep were euthanased by intravenous injection of barbiturate and exsanguination. The lungs were carefully removed and weighed after trimming away the heart, major blood vessels and associated lymph nodes. Macroscopic normality of the lungs was assessed objectively. Thereafter a cannula was securely inserted into the left major bronchus and this lung was inflated with 4% paraformaldehyde in phosphate buffered saline (pH 7.2-7.4). The inflation pressure was maintained at 2.5-3.0 kPa during the fixation period (4 days). The delay between euthanasia and inflation fixation varied between 20 and 380 minutes. Each sheep was randomly allocated to a particular time delay between euthanasia and inflation fixation. During the delay period the lungs were kept at a constant temperature ($4^{o}C$) in the collapsed state. Following fixation the volume of the left lung (TLVF) was determined by water displacement (Aherne & Dunnill, 1982).

6.3 STATISTICAL ANALYSIS

The respective means of three and five determinations of $V_{A,eff}$ and TLV_F were used in statistical analysis. Spearman's rank-order correlation coefficient (r_s) was used to examine the significance of the relationship between variables and Mann-Whitney U tests were used to test the independence of groups. Commercially available statistical software was used in data analysis (Systat for Windows, v.5; Systat Inc., Evanston, IL, USA). .

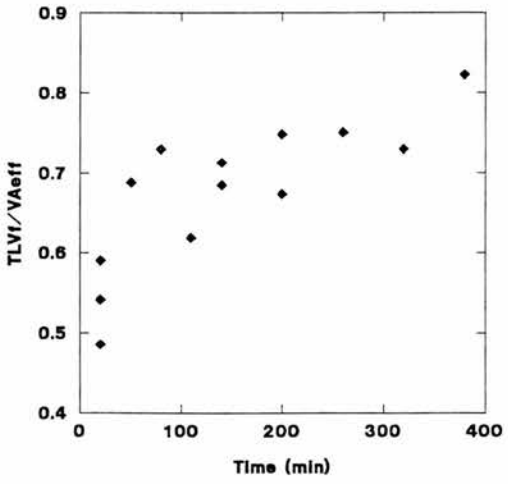
6.4 RESULTS

Values of $V_{A,eff}$, total lung weight (TLW), TLV_F, the time betwixt euthanasia and inflation fixation and the derived ratio of fixed to physiological lung volume ($TLV_F/V_{A,eff}$) are given in table 6.1.

Sheep No.	$V_{A,eff}$	TLW	TLV_F	Time	$TLV_F/V_{A,eff}$	Age Group
	ml	kg	ml	min	-	-
CON133	2508	0.445	1726	50	0.69	1
CON136	2586	0.388	1887	80	0.73	1
CON111	2162	0.560	1338	110	0.62	1
CON114	2374	0.600	1626	140	0.68	1
CON138	2377	0.415	1777	200	0.75	1
CON132	2349	0.400	1763	260	0.75	1
CON140	2280	0.445	1876	380	0.82	1
CON101	3937	0.590	2323	20	0.59	2
CON103	4418	0.590	2144	20	0.49	2
CON105	4299	0.700	2326	20	0.54	2
CON145	4122	0.733	2939	140	0.71	2
CON142	3723	0.810	2508	200	0.67	2
CON144	3487	0.687	2545	320	0.73	2

Table 6.1 Values of $V_{A,eff}$, total lung weight (TLW), TLV_F, the time betwixt euthanasia and inflation fixation and the derived ratio of fixed to physiological lung volume ($TLV_F/V_{A,eff}$).

The relationship between $TLV_F/V_{A,eff}$ and the time betwixt euthanasia and inflation fixation is demonstrated in Figure 6.1. (right) This relationship is significant (r_s = 0.831; P<0.001 (one-tailed)).



The independence of the two age groups in terms of lung volume is demonstrated in appendix 6.2. There is a significant difference between the groups (P<0.005). There is no significant difference between the two groups in terms of the $TLV_F/V_{A,eff}$ ratio (P = 0.063) (Appendix 6.3) although a trend towards a lower ratio in the older age group is demonstrated. When split into respective age groups, significant

positive correlations between $TLV_F/V_{A,eff}$ and the time betwixt euthanasia and inflation fixation could still be demonstrated (r_s = 0.714, P = 0.05 and r_s = 0.880, P < 0.05 for age groups 1 and 2 respectively).

6.5 DISCUSSION

Quantitative microscopic evaluation of normal and diseased tissue states has benefited from advances in morphometric techniques, specifically the development and use of test grids of points, lines or areas for measuring volume fractions, surface areas or counting discrete structures. By defining structural indices for measurement by these techniques, information regarding component substructure, whether this concerns cell or tissue morphology or biology, is not necessarily specifically analysed. However, the use of these techniques by microscopists has initiated a welcome change in the way results are expressed, from a purely descriptive towards a truly quantitative manner. More recently, within the last decade, the advent of sophisticated computer aided technology to acquire, display and analyse microscopic images has brought hitherto inconceivable accuracy to morphometric analysis.

However, with such accuracy available at the analytical stage, it is vital that material subject to analysis is prepared following standardized and accepted protocols i.e. that variability associated with sample preparation for analysis is minimized. The underlying objective of all methods of fixation is (a) to produce material for evaluation that is compatible with the equivalent images of living eukaryotic cells and of tissue structure as they have been worked out in cell biology, and (b) to produce this material in a reproducible and predictable manner (Weibel, 1984).

In this regard, constant-pressure airway inflation fixation was initially proposed by Heard (1958) as a method that would prevent lung shrinkage during fixation and lead to retention of anatomical features. With the advent of quantitative analysis techniques the process of fixing lungs in a controlled state of inflation was of specific value as it provided material that could be interpreted within the context of comparative and physiological studies.

Since its inception, constant-pressure inflation fixation has proved very popular in the scientific literature, providing satisfactory lung inflation whilst being technically undemanding. Given that fixed lung volume is essential for morphometric determination of tissue densities, surface areas, and volumes of air and tissue compartments of the lung (Hayatdavoudi *et al.*, 1980), it is intuitively obvious that such techniques of inflating the lungs must provide highly reproducible results if the benefits of sophisticated image analysis tools are to be realised.

However, Lum & Mitzner (1985) highlighted considerable interspecies variability in lung tissue shrinkage during fixation by constant-pressure airway instillation. These authors compared the effects of 10% formalin fixation on fixed lung volume in several species using pre-fixation measurements of total lung capacity (TLC) as a within subject basis for comparison. Lum & Mitzner (1985) discuss the possible reasons for variability of the fixed to physiological lung volume ratio under standard conditions of measurement and comment on the possible influence of alveolar size, the quantity of collagen and elastin in the lung and the fixative flow rate. In their comprehensive study, Lum & Mitzner (1985) found the ratio of fixed to physiological lung volume in the sheep to be 0.83. Previous experience, using similar techniques, suggested a considerably lower ratio (Chapter 5; Table 5.4.3).

To account for inter-study variability Lum & Mitzner (1985) suggest that differences in fixative and/or flow rate and methods used in determining TLC pre-fixation may increase interstudy variability in the fixed to physiological lung volume ratio. Subjective observations were that delay between euthanasia and lung fixation led to increased fixed lung volume and an increase in the fixed to physiological lung volume ratio ($TLV_F/V_{A,eff}$).

The results of the present study confirm these observations. There was a striking correlation between the $TLV_F/V_{A,eff}$ ratio and the time delay between euthanasia and lung fixation. Although the age and lung size grouping was dichotomous there was no significant difference between the groups in terms of the $TLV_F/V_{A,eff}$ ratio and correlations of this ratio with time remained significant for the individual age groups. Thus the time delay betwixt euthanasia and inflation fixation of sheep lungs influences the ratio of fixed to physiological lung volume. This observation has obvious bearing on interpretations of morphometric analyses in sheep. It is suggested that, where possible, the time between euthanasia and inflation fixation is controlled or else morphometric data corrected to account for variable lung shrinkage during the fixing process.

As to possible mechanisms to account for these observations it is of interest that Lum & Mitzner (1985) experienced difficulty in inflating excised sheep lungs with air and suggested that, as is thought to occur in guinea pig (Lai *et al.*, 1984), post-mortem bronchoconstriction might account for this phenomenom. Lai *et al* (1984) were able to demonstrate bronchiolar constriction that had occurred in guinea pig lungs subjected to inflation fixation and a similar mechanism might account for the observations of Berend *et al.* (1981) in humans, that small airway dimensions were smaller in surgically resected lobes compared to autopsy lungs.

Such a mechanism could explain observations made during this study. If sheep lungs are fixed during a period of intense bronchoconstriction it is likely that (a) the compliance of the lungs will be reduced to the extent that a given inflation pressure of fixative will be less capable of causing expansion, and (b) the smooth muscle causing the constriction will be fixed in a variable state of contraction. Presumably, with time after euthanasia the strength of bronchoconstriction wanes, leading to progressively easier lung inflation. Lai *et al.* (1984) found that blood volume had a marked effect on the onset of bronchoconstriction in guinea pigs in that the presence of blood in the lung protected against bronchoconstriction whereas oxygen availability, temperature, lung innervation, and degree of lung inflation had no influence. Comments regarding the role of the blood were speculative suggesting that the blood perhaps diluted a mediator, accelerated its degradation, inhibited its synthesis, or provided an antagonist (Lai *et al.*, 1984). Hayatdavoudi *et al.* (1980) also made the observation, in rat lungs fixed under constant-pressure, that circulating blood in the lungs at the time fixation was initiated increased the final fixed lung volume. The sheep in this study were exsanguinated immediately after intravenous injection of barbiturate. The degree of exsanguination achieved will presumably vary and must represent another potential influence on the fixed to physiological lung volume.

Further studies on the post-mortem mechanical behaviour of sheep lungs are required to elucidate the mechanisms responsible for the association betwixt the delay between euthanasia and lung fixation and the increase in the fixed to physiological lung volume ratio ($TLV_F/V_{A,eff}$).

6.6 SUMMARY

The influence of time between euthanasia and lung fixation on the fixed to physiological lung volume ratio was investigated in 13 normal sheep lungs. Lungs were fixed by constant-pressure airway instillation of 4% paraformaldehyde for a period of 4 days. The time delay prior to fixation varied from 20 to 380 minutes. The fixed to physiological lung volume ratio was significantly positively correlated with the time delay (r_s = 0.831; P<0.001). Post-mortem bronchoconstriction may account for this phenomenom.

CHAPTER 7

PATHOPHYSIOLOGICAL CORRELATIONS IN MAEDI

7.1 INTRODUCTION

Lymphoid interstitial pneumonia (LIP) is characterized by the interstitial accumulation of lymphocytes, macrophages and plasma cells. In humans, this condition may accompany several immunologically mediated diseases and also occurs in association with HIV infection, particularly in children (Teirstein & Rosen, 1988). No clinical feature or non-invasive test can specifically confirm the presence of LIP and lung biopsy is the only means of establishing a definitive diagnosis of this condition (Vath *et al.*, 1982)).

Physiologically, the human interstitial lung diseases are generally characterized by a reduction in lung volumes, lung compliance and diffusing capacity (Wanner, 1980). Serial measurements of lung function indices are utilized in the assessment and management of LIP (Popa, 1988). However, attempts at relating functional abnormalities to histologic indices of disease activity in interstitial lung disease are often disappointing (Gaensler et al., 1975; Green *et al.*, 1976) with functional indices roughly reflecting the overall pathology but being relatively insensitive. This insensitivity may in part be due to both sampling problems i.e. extrapolating morphological data from a biopsy specimen to the whole lung (Gaensler et al., 1975), and to temporal separation of the functional tests from necropsy examination of the lung (Lavietes *et al.*, 1977).

If animal models of human disease are used however, then an accurate qualification and quantification of pulmonary pathology can be made at necropsy examination. Correlation of this data with functional measurements made immediately prior to euthanasia allows a more accurate assessment of the specificity of such measurements to be made. In sheep, natural and experimental infection with maedi-visna virus (MVV), causes a chronic progressive lymphoproliferative disease which affects the lungs, nervous system, mammary glands and joints (Pálsson, 1976; Lairmore *et al.*, 1986). The respiratory pathology of this disease is similar to that described for LIP associated with HIV infection in humans (Lairmore *et al.*, 1986). Thus, an appropriate animal model for LIP, particularly that associated with HIV infection, exists in sheep, an animal with lungs of comparable size and similar structure to those of human beings and in which the lung functional abnormalities associated with MVV infection have been characterised (Chapter 4).

Knowledge of how functional measurements in LIP associated with MVV-infection specifically relate to structural indices of disease severity will be of value in staging and managing the disease in sheep and will be particularly relevant to the assessment of therapeutic and manipulative strategies in this condition. This chapter describes the objective quantitative assessment of maedi and presents the relationships that exist between functional and structural indices in this condition.

7.2 MATERIALS AND METHODS

7.2.1 Animals

16 adult Texel ewes (median body weight, 57.5 kg; range, 49 to 67 kg), seropositive for MVV on the basis of results of the agar gel immunodiffusion test (Winward *et al.*, 1979), were used in this study. These sheep were selected from a flock, the history of which has previously been described (Watt *et al.*, 1992). Sheep were housed during the period of physiologic study. Neither clinical nor routine haematological examinations indicated the presence of significant respiratory disease unrelated to infection with MVV. Subsequent necropsy examination confirmed the absence of significant respiratory disease unrelated to MVV-infection in these sheep.

7.2.2 Clinical studies

A subjective clinical grading of severity of respiratory disease was made on each sheep. A grade of 0 was assigned when there was no clinical evidence of respiratory disease; a grade of 1, when the only evidence of respiratory disease was an increase in adventitious sounds on auscultation; and a grade of 2 when there was, in addition, evidence of dyspnoea with or without mouth breathing. Body condition scores were assessed on a scale of 0 to 5 with 0 representing extreme emaciation and 5 excessive fat cover (Meat and Livestock Commission, 1981).

7.2.3 Physiologic studies

The precise methodology employed to measure effective alveolar lung volume ($V_{A,eff,}$) single-breath transfer factor for carbon monoxide ($T_{L,CO,'sb'}$) and transfer factor corrected for lung volume ($T_{L/VA}$) has been described previously (Chapter 2; section 2.2.3). Three determinations of lung volume and transfer factor were made on each sheep, and the mean value was calculated. Measurements of static lung compliance (Cst) were executed as previously described (Chapter 2; section 2.2.3). An average of 41 data points (range 20 to 56) were collected from each animal. Static lung compliance (Cst) was measured on the expiratory limb of static pressure-volume curves between functional residual capacity (FRC) and 40% of the way from FRC to total lung capacity (TLC) i.e. the lung volume at a Ptp of 3 kPa. The mean of three Cst determinations was calculated. Exponential curve fitting analysis was carried out as described in chapter 3 (3.2.5).

7.2.4 Tissue Preparation

Euthanasia and removal, assessment and fixation of lung tissue was carried out as previously described (Chapter 5, sections 5.2.2-5.2.3).

7.2.5 Morphometric Analyses

The volume of the fixed left lung was determined by water displacement as previously described (Chapter 5, section 5.2.4) and the total lung volume (TLV_F) was inferred from the ratio of left lung weight to total lung weight. The lung was sliced into 1cm thick transverse slices and six slices were selected for tissue sampling from the region just caudal to the cranial margin of the ventral basal segment of the diaphragmatic lobe (Hare, 1955). Sampling of tissue blocks, histologic processing and staining, selection of fields and morphometric analysis were executed as previously described (Chapter 5.2.5-5.2.6). The variables measured were volume densities of tissue (V_{vt}) and air (V_{va}) and the alveolar surface density (S_{vt}). The parenchymal fraction of the total volume was estimated as 0.85, based on point counting of lung slices (Chapter 5, section 5.4). Since all lungs were treated in an identical manner, it was considered unnecessary to calculate fixation or processing constants.

7.3 STATISTICAL ANALYSIS

To facilitate discussion of the analysis, data were grouped as follows: (a) clinical data, including age, bodyweight (BW), body condition scores and subjective grading of disease severity; (b) physiological data including Cst, K, $V_{A,eff}$ and $T_{L,CO,'sb'}$ and $T_{L,CO,'sb'}$ values corrected for alveolar volume ($T_{L/VA}$), and (c) morphometric data including lung weight, TLW, TLV_F, V_{vt}, S_{vt} and the absolute parameters total parenchymal airspace volume ($TV_{p,a}$) and total parenchymal airspace surface area (ASA_p) derived from this data. The Spearman-rank correlation coefficient (r_s) was used to compare within and between groups and $P<0.01$ was accepted as an appropriate level of significance. Where a large proportion of tied ranks were present in the data, a correction factor was used to correct the sum of squares (Siegel & Castellan, 1988). Physiological measurements were also expressed as percent predicted values, these values being obtained from previously generated regression equations (Chapter 2). Linear regression analysis was used where appropriate.

7.4 RESULTS

7.4.1 Clinical data

Clinical data are shown in appendix 7.1. 9 sheep had no clinical evidence of respiratory disease (grade 0), 4 demonstrated an increase in adventitious sounds on auscultation (grade 1), and 3 had in addition, more overt signs of respiratory disease (grade 2). BW was positively correlated with

condition score (r_s 0.633; P<0.01), however, neither BW nor condition score were correlated with clinical score.

7.4.2 Morphometric data

Mean values for morphometric data are shown in appendix 7.2. Lung weights ranged from 0.55 to 1.74 kg. Tissue density (V_{vt}) ranged from 17.0 to 50.2% and absolute estimates of total parenchymal airspace volume from 1025 to 2198 ml. Surface density of the alveolar epithelium varied from 243.4 to 684.9 cm^2/cm^3, tissue surface-to-volume ratio from 499.7 to 3426.0 cm^2/cm^3 and total parenchymal airspace surface area from 48.7 to 156.7 m^2.

7.4.3 Physiologic data

Mean values for physiological data are shown in appendix 7.3. Physiological data are illustrated in figures 7.1-7.5. Values are expressed as percentage of predicted values for sheep of the same bodyweight and breed (Chapter 2). The dotted lines in figures 7.1-7.4 represent the 95% confidence limits for normal values for sheep of the same breed and sex (Chapter 2). The majority of sheep (n=12) had Cst values within or above the normal range (Figure 7.1). 7 sheep had $V_{A,eff}$ values in the normal range (Figure 7.2). Although 4 sheep had $T_{L,CO,'sb'}$ values within the normal range (Figure 7.3)(overleaf) only two of these sheep had $T_{L/VA}$ values in the normal range (Figure 7.4)(overleaf).

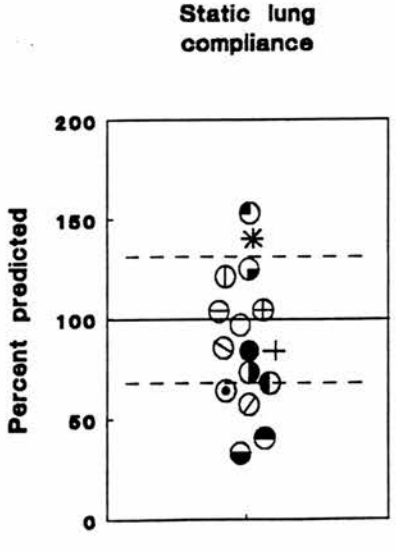


Figure 7.1 Relative distribution of static compliance measurements from 16 sheep seropositive for MVV.

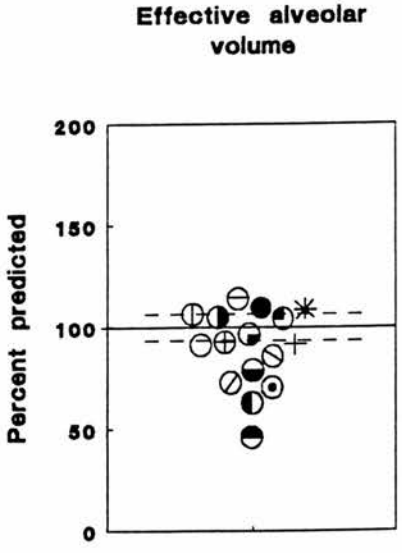


Figure 7.2 Relative distribution of effective alveolar volume measurements from 16 sheep seropositive for MVV.

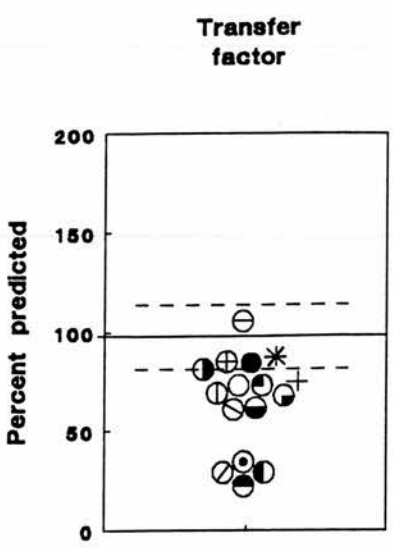


Figure 7.3 Relative distribution of transfer factor measurements in 16 sheep seropositive for MVV.

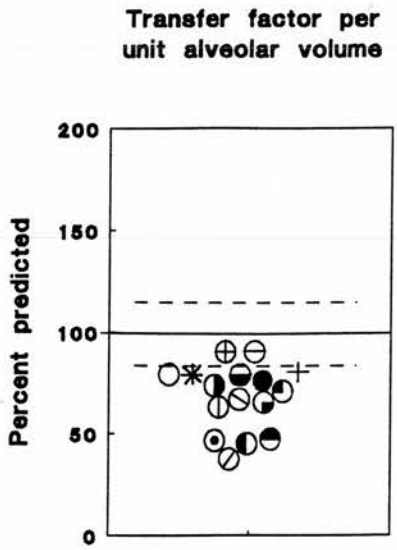


Figure 7.4 Relative distribution of transfer factor measurements corrected for alveolar volume in 16 sheep seropositive for MVV.

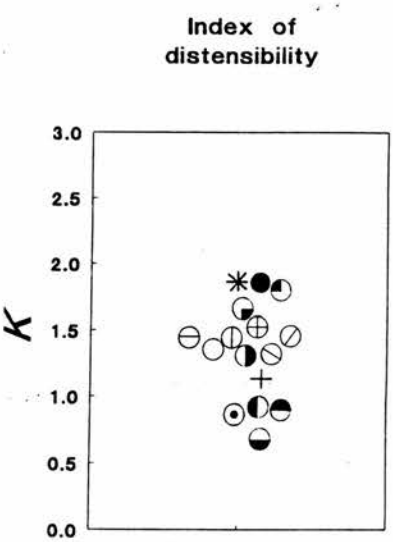


Figure 7.5 Relative distribution of *K* measurements in 16 sheep seropositive for MVV. Dotted lines represent limits of normal range (Chapter 3).

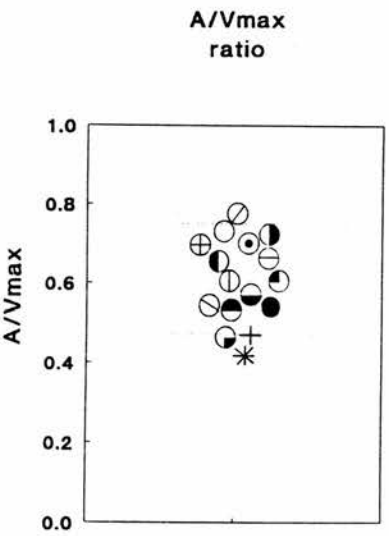


Figure 7.6 Relative distribution of the A/Vmax ratio from measurements made in 16 sheep seropositive for MVV. Dotted lines represent limits of normal range (Chapter 3).

Values of *K* ranged from 0.685 to 1.866 kPa (Figure 7.5)(normal range 1.091 to 2.119 kPa; (Chapter 3) and the median coefficient of determination (r^2) was 99.16% (range 95.27 to 99.83%). The ratio of A/Vmax ranged from 0.420 to 0.774 (Figure 7.6)(normal range 0.482 to 0.754, (Chapter 3). There was no obvious trend towards either an increase or decrease in this ratio.

7.4.4 Comparison of clinical data with morphometric and physiologic data

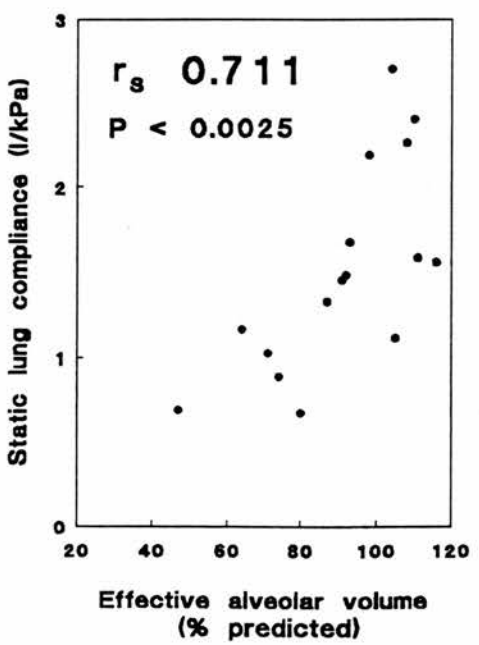
Surface density was positively correlated with BW (r_s 0.662; P<0.005) and body condition score (r_s 0.590; P<0.01). Condition score was negatively correlated with V_{vt} (r_s -0.590; P<0.01). Clinical score was positively correlated with TLW (r_s 0.615; P<0.01). There were no significant correlations between clinical score, condition score and BW, and the physiological data. Results of Spearman rank correlation tests between clinical and morphometric and physiologic data are shown in appendix 7.4.

7.4.5 Comparisons between morphometric parameters

Notable correlations were the strong negative correlation between TLW and S_{vt} (r_s -0.693; P<0.0025) and the strong positive correlation between $TV_{p,a}$ and total gas exchange surface area (r_s 0.826; P<0.0005). TLV_F was not significantly (P>0.01) related to V_{vt}, S_{vt} or total gas exchange surface area. Surface density was negatively correlated with V_{vt} (r_s -0.676; P<0.005) and positively correlated with total gas exchange surface area (r_s 0.591; P<0.01). The above relationships are illustrated in appendices 7.5.1-7.5.4. All correlations between morphometric variables are given in appendix 7.6.

7.4.6 Comparisons between the various physiologic variables

Static lung compliance was positively correlated with K (r_s 0.800; P<0.0005), $T_{L,CO,'sb'}$ (r_s 0.726; P<0.0025) and $V_{A,eff}$ (r_s 0.765; P<0.001), and K was also positively correlated with $V_{A,eff}$ (r_s 0.706; P<0.0025). Figure 7.7 (right) demonstrates the relationship between $V_{A,eff}$ (expressed as percent of predicted value) and Cst (r_s 0.729; P<0.0025), and figure 7.8 (overleaf) demonstrates the relationship between $V_{A,eff}$ (% predicted) and K (r_s 0.694; P<0.0025) for the whole data set. The correlation coefficients and levels of significance given in figures 7.7 & 7.8 refer to the results of analyses from which sheep 89 was excluded (see 7.4.7 & discussion). Figure 7.9 (overleaf) illustrates the significant positive correlation between $T_{L,CO,'sb'}$ and $V_{A,eff}$ (r_s 0.882; P<0.0005). All correlations between physiological variables are given in appendix 7.7.



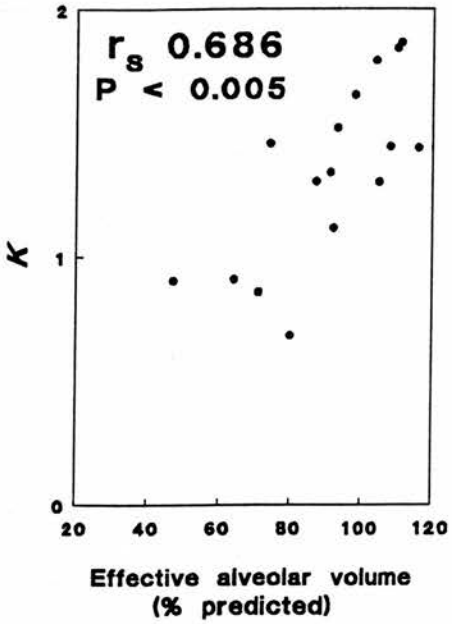


Figure 7.8 Relationship between lung distensibility (K) and effective alveolar volume ($V_{A,eff}$) expressed as percent predicted value.

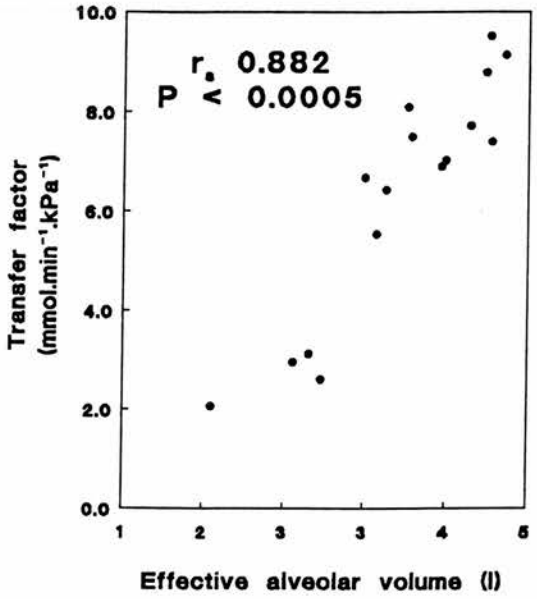


Figure 7.9 Relationship between transfer factor ($T_{L,CO,'sb'}$) and effective alveolar volume ($V_{A,eff}$).

7.4.7 Comparison of morphologic with physiologic data

Correlation coefficients and levels of significance of the relationship between morphologic and physiologic variables are shown in table 7.1 (below). In the initial analysis, no significant

Table 7.1

	TLW	TLV_F	V_{vt}	$TV_{p,a}$	S_{vt}	ASA_p
Cst	-0.582 P<0.01	-0.324 NS	-0.632 P<0.01	0.203 NS	0.585 P<0.01	0.332 NS
K	-0.555 NS	-0.185 NS	-0.532 NS	0.288 NS	0.450 NS	0.268 NS
A/Vmax	0.274 NS	0.436 NS	0.327 NS	0.107 NS	-0.359 NS	0.057 NS
$T_{L,CO,'sb'}$	-0.595 P<0.01	-0.088 NS	-0.762 P<0.001	0.582 P<0.01	0.656 P<0.005	0.694 P<0.0025
$T_{L/VA}$	-0.657 P<0.005	-0.279 NS	-0.721 P<0.0025	0.426 NS	0.576 NS	0.524 NS
$V_{A,eff}$	-0.515 NS	-0.047 NS	-0.653 P<0.005	0.591 P<0.01	0.700 P<0.0025	0.779 P<0.0005

correlations were demonstrated between K and the morphometric variables (table 7.1), however one K value (sheep 89) was noted to have disproportionate influence and this was removed (see

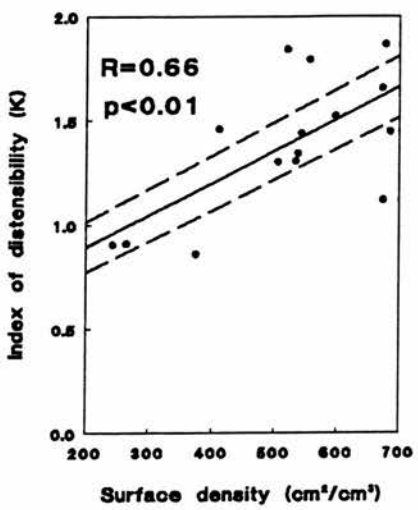
Table 7.2

	TLW	TLV_F	V_{vt}	$TV_{p,a}$	S_{vt}	ASA_p
K	-0.829 P<0.0005	-0.318 NS	-0.764 P<0.001	0.346 NS	0.579 P<0.025	0.325 NS

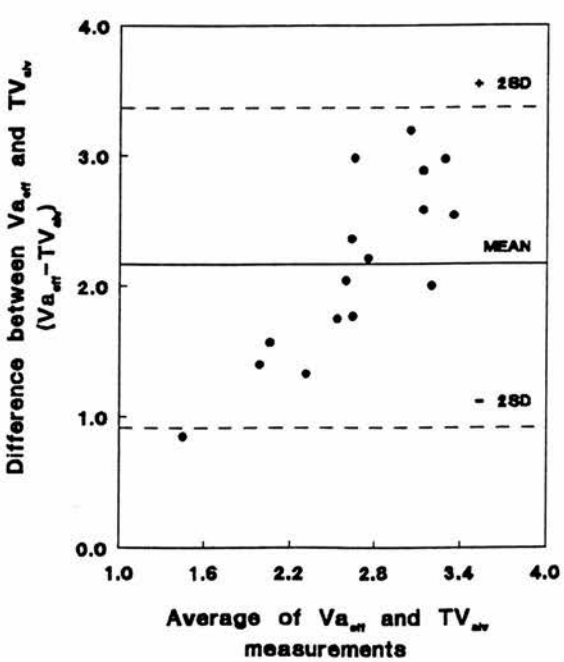
discussion). Subsequent analysis of the reduced data set (n=15) reveals highly significant correlations between K and TLW, and K and V_{vt} (table 7.2 above). These relationships are demonstrated in appendices 7.8 and 7.9. The correlation between K and S_{vt} was only just non-significant ($0.01<P<0.025$). Linear regression of K on S_{vt} (cm^2/cm^3) gave the equation

$$K = 0.585 + (0.00154 \cdot S_{vt})$$

which was significant ($R = 0.66$; $P<0.01$). The relationship is demonstrated in figure 7.10 (right).



Although morphometric estimates of $V_{A,eff}$ are significantly correlated with the physiologic measurements of the same, it is evident that there is poor agreement between these two measurements (Figure 7.11, right), with physiologic estimates considerably overestimating morphometric (median difference 2.12l; range 0.85 - 3.19l). The fixed to physiological lung volume ratio ($TLV_F/V_{A,eff}$) ranged from 0.440 to 1.253 (median 0.692) and the ratio of total parenchymal airspace volume to physiological



lung volume ($TV_{p,a}/V_{A,eff}$) ranged from 0.281 to 0.553 (median 0.442) (Appendix 7.10).

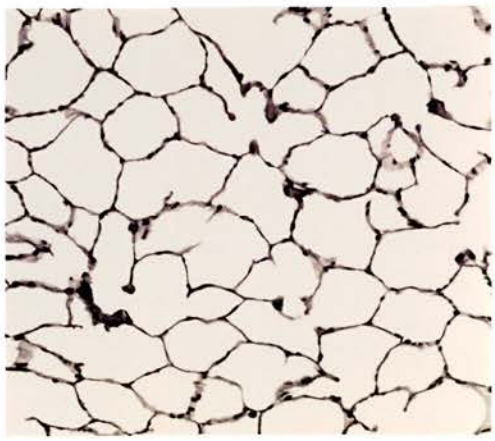
7.5 DISCUSSION

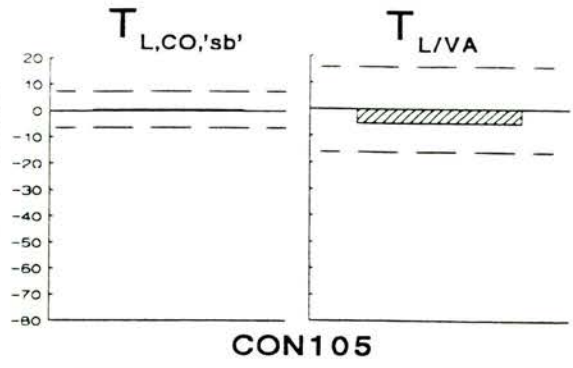
It was demonstrated in chapter 4 that sheep with maedi have significantly reduced lung volumes and transfer factor values, increased elastic recoil and a trend towards reduced static lung compliance compared to control sheep. In this study, the intention was to select sheep demonstrating a wide spectrum of clinical and functional abnormality such that the structural basis behind these observations could be investigated. The results of this study indicate that (a) abnormalities of diffusion and gas exchange occur prior to significant structural pathology and are a sensitive means of assessing this disease, and (b) that changes in the elastic behaviour of the lungs, which cannot be explained by surface related forces, may be related to parenchymal smooth muscle hyperplasia, a pathological feature of maedi.

The range of lung pathology represented was wide with TLW varying from 0.55 to 1.74 kg. Based on the power law formulae of Stahl (1967), the normal range of TLW for sheep of the same BW as the sheep used in this study is 0.53-0.73kg. Correlations between S_{vt} and V_{vt}, and body condition score and BW indicated that sheep with less structural abnormalities present in their lungs tended to be in better condition.

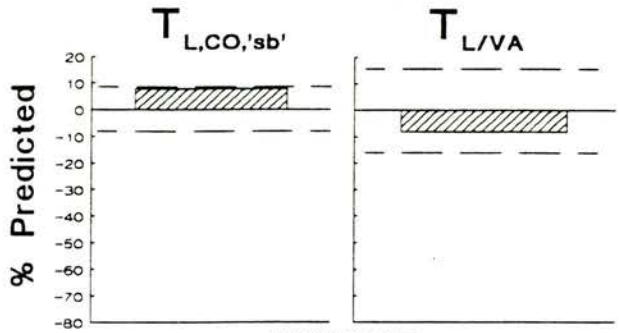
A larger proportion of sheep had abnormally low $T_{L,CO,'sb'}$ and $T_{L/VA}$ values than had low $V_{A,eff}$ or Cst values, and reduced values of $T_{L,CO,'sb'}$ and $T_{L/VA}$ occurred in several sheep that had little or no evidence of structural abnormality (Table 7.1, Figure 7.3 &7.4). These observations are illustrated overleaf (Figure 7.12). This reflects clinical experience with human interstitial lung disease where, of the functional measurements, transfer factor and gas exchange studies appear to be the most sensitive indicators of alveolitis in both human idiopathic fibrosis and sarcoidosis (Miller *et al.*, 1976). Further, it is of note that $T_{L/VA}$ is strongly negatively correlated with TLW and V_{vt} (Table 7.1) suggesting that as disease progresses, in addition to volume related effects, factors associated with the development of the interstitial reaction such as regional inhomogeneities in the lungs regarding blood flow, ventilation or gas diffusing properties, reduced pulmonary capillary blood volume or thickening of the alveolar capillary membrane are important in limiting gas diffusion.

Figure 7.12 (facing) Representative photomicrographs of lung histology and histopathology from 4 sheep. All except CON105 were seropositive for MVV. The extent of functional disturbances in transfer factor and volume corrected transfer factor are shown adjacent to the micrographs. Lung histology from MVV017 was essentially normal and this finding was reflected in functional studies. MVV007 is an example of a sheep with minimal or no evidence of histopathology but with significant functional deficit and MVV068 was an example of a sheep with no clinical evidence of respiratory disease (Appendix 7.1), yet with significant functional deficit and obvious histopathology.

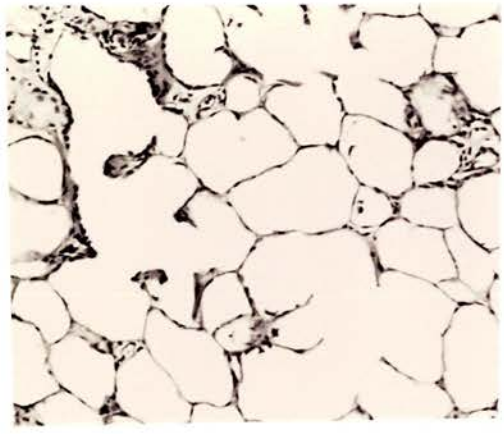


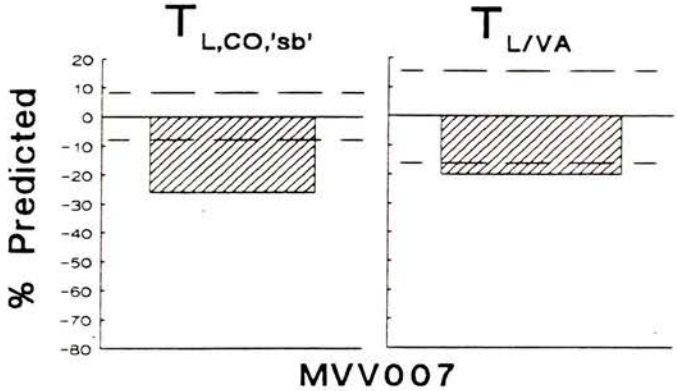


CON105

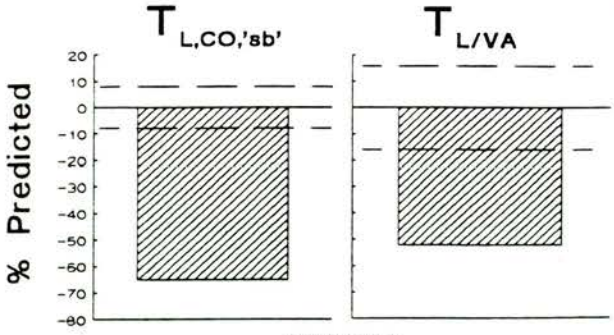


MVV017

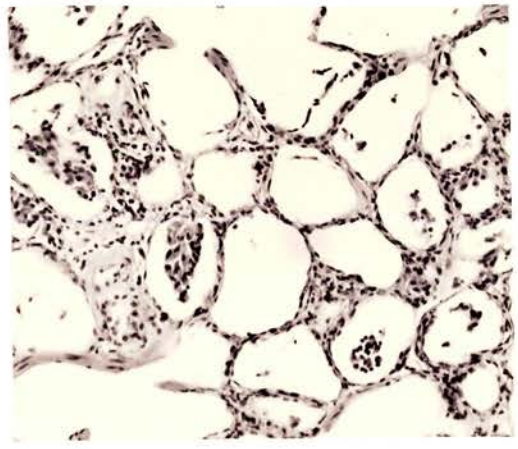




MVV007



MVV068



Some HIV-infected individuals with no detectable lung infection or tumour have respiratory symptoms and/or abnormalities in gas exchange (Guillon *et al.*, 1988). A cytotoxic CD8 lymphocytic alveolitis is frequently identified in these subjects and seems to correlate with abnormal lung function indices (Wasserman *et al.*, 1991). Meignan *et al.* (1990) demonstrated that a reduction in $T_{L,CO,'sb'}$ and $T_{L/VA}$ was associated with an increase in lung epithelial permeability in these patients and that the increase in permeability was highly correlated with HIV-specific cytotoxic activity. This suggested that some immunologic conflict between HIV-specific cytotoxic T-lymphocytes and HIV-infected target cells may be responsible for damage to the lung epithelium (Meignan *et al.*, 1990). In naturally occurring maedi there is also a CD8 T-lymphocytic alveolitis (Cordier *et al.*, 1992; Luján *et al*, 1993). It is therefore possible that similar mechanisms may be responsible for the impaired gas exchange apparent in those sheep infected with MVV but showing negligible structural pathology.

Subjective and morphometric assessment suggested no pathologic change in sheep 89. It was therefore surprising that Cst and *K* values were so low in this animal. This anomalous observation presumably reflected a dynamic influence peculiar to this sheep during the measurement period, such as variation in the smooth muscle tone surrounding the airways, vasculature or parenchyma or variation in pulmonary blood volume. In that this sheep did not seem representative of the structure-function relationships otherwise demonstrated we felt justified in removing it from any analyses concerning Cst and *K* measurements.

There was considerable disagreement between the respective estimates of alveolar volume ($V_{A,eff}$ and $TV_{p,a}$)(Figure 7.11). An additional observation was that the difference was not consistent and tended to increase as alveolar volume estimates increased. Examination of the fixed to physiological lung volume ratios (Appendix 7.10) revealed a wide range of values and comparison with ratios obtained from control sheep (chapter 5)(0.49-0.59) revealed that the majority exceeded those of the controls. Indeed the ratio of fixed to physiological lung volume, in line with the observed disagreement between $V_{A,eff}$ and $TV_{p,a}$, was significantly positively correlated with total lung weight (Figure 7.13)(r_s 0.847; P<0.005), a useful gross index of disease progression. One possible cause of variation in this ratio is variation in the time between

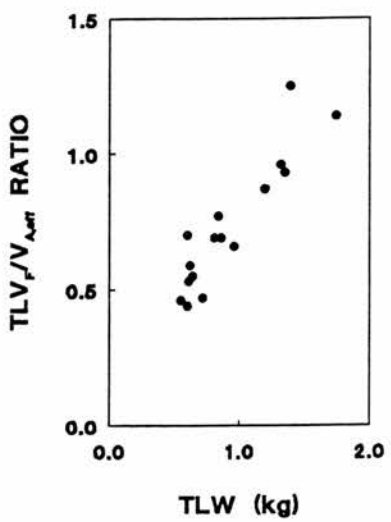


Figure 7.13 The relationship between TLW and the $TLV_F/V_{A,eff}$ ratio.

euthanasia and inflation fixation of the lungs (chapter 6), however, given the standardized time between euthanasia and fixation in this study, it is interesting to consider which aspects of the disease process may contribute to variable tissue shrinkage.

The relative quantities of collagen and elastin fibres i.e. "fixable and nonfixable" connective tissue (Lum & Mitzner, 1985), in the pleura and alveolar septae may influence fixed lung volume, in that following release of the inflating fixative, the elastin, which cannot be fixed (Sobin *et al.*, 1982), may cause the lungs to recoil. It is interesting to note that Georgsson *et al.* (1976) described elastic fibres to be sparse and fragmented in maedi. This fragmentation would presumably decrease recoil post-fixation and lead to a relative overestimate of the fixed lung volume, in turn leading to increased fixed to physiological lung volume ratios as disease progresses i.e. the observed relationship in this study.

An alternative to the hypothesis that tissue shrinkage is responsible for variation in the fixed to physiological lung volume ratio is that *in vivo* lung mechanical properties are altered in disease and limit the physiological lung volume achieved during inflation to a Ptp of 3kPa. In line with this alternative hypothesis is the observation that, in this study, there is an abnormal reduction in $V_{A,eff}$ and that this reduction is associated with a reduction in Cst, and in chapter 4, in a larger data set, a significant increase in elastic recoil was observed in seropositive sheep. The positive correlation between percent predicted $V_{A,eff}$ and K indicates that the distensibility of remaining inflatable lung units is progressively reduced as lung volume is reduced i.e. as disease progresses. Thus, diseased lungs are less distensible in LIP associated with MVV-infection and this reduction in distensibility may contribute to limitation of the lung volume achieved at a given inflating pressure, leading to an increase in the fixed to physiological lung volume ratio (Figure 7.13). The question then arises as to whether tissue or surface related components are responsible for this reduced distensibility.

Haber *et al.* (1983) demonstrated that, in excised mammalian lungs (rat, cat and dog), the density of surface forces was the major determinant of lung distensibility, with distensibility being significantly related to peripheral airspace size (Appendix 7.11). However, it would appear that in maedi, there is a positive correlation between S_{vt} and K (figure 7.10). Therefore the lack of distensibility in maedi is not explained by the density of surface forces. The positive correlation and significant linear relationship between the density of surface forces and K is in fact the exact opposite to perceived relationships that occur in association with advancing age (Colebatch & Ng, 1986) and with fibrosing alveolitis (Thompson & Colebatch, 1989) in humans. With advancing age, an increase in the size of airspaces is associated with an increase in K (Colebatch & Ng, 1986), and

in fibrosing alveolitis it is thought that there is a reduction in the size of airspaces which in turn results in a reduction in *K*. It therefore appears that these findings are at variance with previous authors (Haber *et al.*, 1983; Colebatch & Ng, 1986; Thompson & Colebatch, 1989) who attribute variations in *K* to differences in the density of surface forces. That there is indeed a progressive increase in airspace size with disease progression is evinced by the photomicrographs overleaf (Figure 7.14)(all at x100) which demonstrate histology from control lungs (CON101) through progressively more advanced histopathology (MVV053 & MVV079). The increase in airspace size with disease progression is obvious.

One therefore asks whether there are dynamic tissue forces present *in vivo* which are capable of influencing *K* but whose influence is removed during the fixation process? It is tempting to speculate as to possible causes of the observed variance in *K* that are only present *in vivo*.

A notable pathological feature of maedi is a pronounced increase in contractile tissue within the pulmonary parenchyma, both at the level of the alveolar ducts and respiratory bronchioles and also extending into the interalveolar septae (Georgsson *et al.*, 1976). In normal lungs, in addition to the smooth muscle in the alveolar ducts and in the walls of the pulmonary vessels and airways there is a variable amount of contractile tissue present in the pulmonary interstitium (Evans *et al.*, 1983) which may substantially increase in amount in natural (Adler *et al.*, 1981,1989) and experimental fibrotic lung disease (Adler *et al.*, 1986). The potential contribution of parenchymal contractile tissue to the overall mechanical properties of the lungs is implied by both *in vitro* (Colebatch *et al.*, 1971; Kapanci *et al.*, 1974; Evans *et al.*, 1981,1982) and *in vivo* studies (Nadel *et al.*, 1964; Colebatch *et al.*, 1966). *In vivo* observations of rapid and reversible changes in lung elastic recoil in response to β-2 agonist administration (DeTroyer *et al.*, 1978) and in association with exercise-induced dyspnoea (Hudgel *et al.*, 1976; Peress *et al.*, 1976) in humans provides further challenge to the traditional view that the elastic properties of the lung parenchyma are fixed, static and virtually immutable properties which do not change rapidly with time.

Given that parenchymal smooth muscle hyperplasia is a feature of maedi, a potential mechanism for tissue related forces to alter the lung elastic properties exists. Indeed it has been suggested that the increase in contractile tissue seen in MVV-infection is a compensatory response for the loss of elastic recoil as a result of destruction of elastic fibres in this disease (Georgsson *et al.*, 1976). That contractile tissue tone within the parenchyma might be responsible for variation in *K in vivo* is an attractive hypothesis that deserves further study.

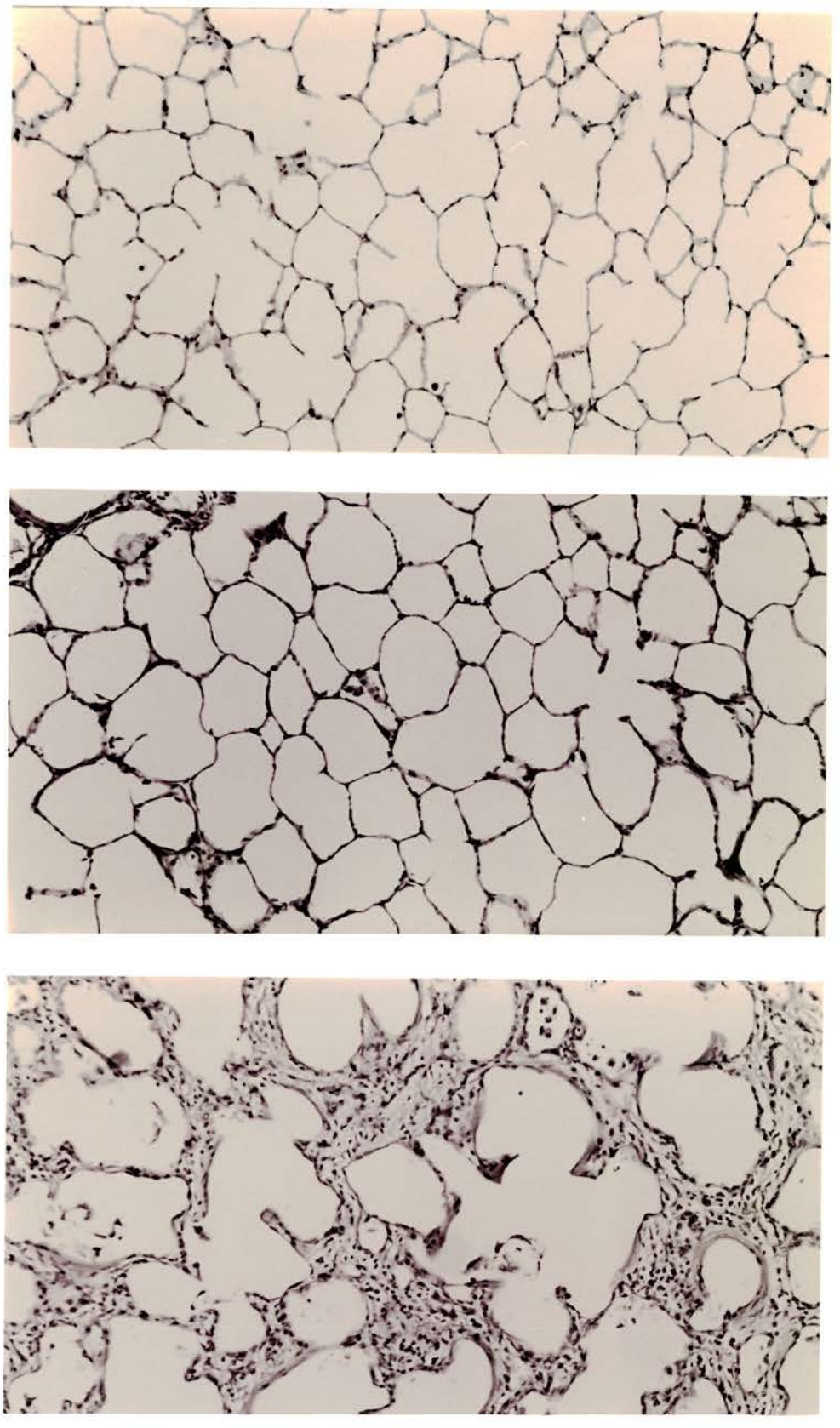


Figure 7.14 Illustrating the increase in airspace size with advancing maedi. Top - control lung (CON101); Middle - lung from seropositive sheep, moderate pathology (MVV053), Bottom - lung from sheep with advanced pathology (MVV079)(all at x100).

Other potential sources of variability in K concern airway and vascular elements, in that substantial changes in lung elasticity result from contraction of the larger airways in anaesthetized sheep (Mitzner *et al.*, 1992) and it is known that variation in pulmonary blood volume will influence lung elasticity (Agostoni & Hyatt, 1986). In the latter instance any changes are likely to be mimimal as even large experimental changes of pulmonary artery pressure and pulmonary blood flow leave the pressure-volume relationship of dog lungs virtually unaffected (Borst *et al.*, 1957).

7.6 SUMMARY

Effective alveolar volume ($V_{A,eff}$), transfer factor for carbon monoxide ($T_{L,CO,'sb'}$), volume corrected transfer factor ($T_{L/VA}$), static lung compliance (Cst) and lung distensibility (K) were measured in 16 sheep seropositive for Maedi-visna virus (MVV) immediately prior to euthanasia. Lungs were inflation fixed and the left lung was randomly sampled for morphometric analysis. The total lung weight (TLW), total fixed lung volume (TLV_F), volume densities of tissue (V_{vt}) and air (V_{va}) and the alveolar surface density (S_{vt}) were measured and correlated with the physiological measurements. TLW and V_{vt} were increased more than twofold and S_{vt} reduced to half of normal in sheep with clinically obvious disease. Of the physiological values, $V_{A,eff}$, $T_{L,CO,'sb'}$ and $T_{L/VA}$ values were less than predicted in the majority of the sheep. Strong correlations between physiological and pathological measurements were demonstrated. In general, measurements of $T_{L,CO,'sb'}$, $T_{L/VA}$ and $V_{A,eff}$ were more sensitive indices of pathology than measurements of Cst or K. Transfer factor measurements were volume dependent and correlated well with volume related parameters such as S_{vt}, $TV_{p,a}$ and ASA_p whereas $T_{L/VA}$ was negatively correlated with indices reflecting the extent of interstitial reaction such as TLW and V_{vt} indicating that factors distinct from lung volume reduction are important in limiting gas exchange in maedi. Transfer factor measurement values were reduced even in sheep with minimal or no morphometric evidence of pathology suggesting their value as a sensitive, if relatively non-specific, means of assessing this condition. Tissue shrinkage differed according to the stage of the disease. The density of surface forces could not account for variation in the distensibility of the lungs, however other factors such as the quantity and functional tone of contractile tissue in the parenchyma, airways or blood vessels may contribute to the variation in lung distensibility seen *in vivo*.

CHAPTER 8

DISTRIBUTION AND QUANTITATION OF LUNG PARENCHYMAL CONTRACTILE TISSUE IN MAEDI.

8.1 INTRODUCTION

The elastic properties of the lungs are determined by both surface and tissue related forces. Whereas surface related forces are determined by the surface area and composition of the alveolar lining fluid, tissue related forces are determined by both fibrous and contractile components within the framework of airways, vessels, parenchyma and pleura. Within the parenchyma much of the contractile component is present in the form of histologically identifiable smooth muscle at the level of the alveolar ducts, and contraction of this element can effect a reduction of lung compliance and lung volume (Halmagyi & Colebatch, 1961b; Nadel *et al*, 1964; Colebatch *et al*., 1966; Colebatch & Mitchell 1971; Colebatch & Engel 1974). Pronounced hyperplasia of this parenchymal smooth muscle element is a feature of maedi (Georgsson *et al*, 1976), and in this condition there is a reduction in static lung compliance and lung distensibility (Chapter 7; section 7.4). However, in contrast to demonstrated (Haber *et al*, 1983) and perceived notions (Colebatch & Ng, 1986; Thompson & Colebatch, 1989) of the relationship between lung distensibility and surface density there is a positive correlation between these parameters in maedi (Chapter 7; section 7.4) suggesting that tissue forces, rather than surface related forces, may limit lung distensibility in maedi. Given that parenchymal smooth muscle hyperplasia is a notable feature of maedi (Georgsson *et al*, 1976) it is tempting to hypothesize that this contractile tissue element is the source of these tissue forces.

This chapter describes the distribution and morphometric quantitation of lung parenchymal contractile tissue in normal sheep lungs and in lungs from sheep seropositive for MVV and examines the relationship between the quantity of parenchymal contractile tissue and various indices of lung elasticity.

8.2 MATERIALS & METHODS

8.2.1 Physiologic data

15 adult Texel ewes, seropositive for MVV, and 3 adult Oxford-Texel cross ewes, from an accredited MVV-free source were used in this study. Functional data relating to lung elasticity in these sheep has been reported (Chapters 3 & 7).

8.2.2 Source of material

Between 5 and 8 (median = 6) of the original 12 paraffin-embedded blocks prepared from each lung (Chapters 5 & 7) were randomly selected for subsequent immunocytochemistry and morphometric analysis. After selection, each block was sectioned on a rotating microtome at 7μ.

8.2.3 Immunocytochemistry

Immunocytochemical techniques were directed towards the localization of α-smooth muscle actin (ASMA). Histologic sections were deparaffinized in xylene and rehydrated through graded solutions of ethyl alcohol. The avidin-biotin complex (ABC) immunoperoxidase method (Vector laboratories, Burlingame, CA, U.S.A.) was used for localizing immunoreactivity. Endogenous peroxidase was inactivated by immersing the sections in a 3% H_2O_2 solution for 20 min at room temperature. Sections were treated with 0.1% protease type I (P 4630; Sigma Chemical Company, Dorset, England) in 0.05 M Tris-HCl buffered saline (pH 7.6) for 60 min at 37°C to unmask antigenic sites (Mepham *et al.*, 1979). Primary antibody incubations were performed at 4°C for 18h. Sections were stained with a commercial anti-ASMA murine monoclonal antibody from an ascites source (A 2547; Sigma Chemical Company, Dorset, England). Optimal antibody concentration was determined to be 1.25 μg/ml by serial dilution in phosphate-buffered saline (PBS). Control sections were stained with normal mouse serum at the same concentration as the monoclonal antibody. 3-amino-9-ethylcarbazole (AEC) was used as the chromogen for quantitative immunocytochemistry and diaminobenzidine (DAB) tetrahydrochloride (with nickel chloride added) was used as the chromogen for photomicrographs.

8.2.3 Morphometric Analysis

Fields were selected for microscopic analysis according to the technique described in chapter 5 (section 5.2.6). Point counting, using a 121 point grid eyepiece graticule (NE 35: Graticules Ltd.), was the method employed to quantify the volume density of parenchymal tissue (V_{vt}) and ASMA ($V_{v'ASMA'}$). The number of points counted depended on the volume fraction of the studied components in that the size of the standard error relative to the mean value of the count i.e. the relative standard error (RSE), had to be reduced to less than 5% for each measurement (Aherne & Dunhill, 1982)(chapter 5, section 5.3) to ensure that sampling error was reduced to within acceptable limits. To facilitate calculation of absolute values, a value of 0.85 was assumed for the parenchymal volume fraction (V_{vp})(chapter 5, table 5.4.1).

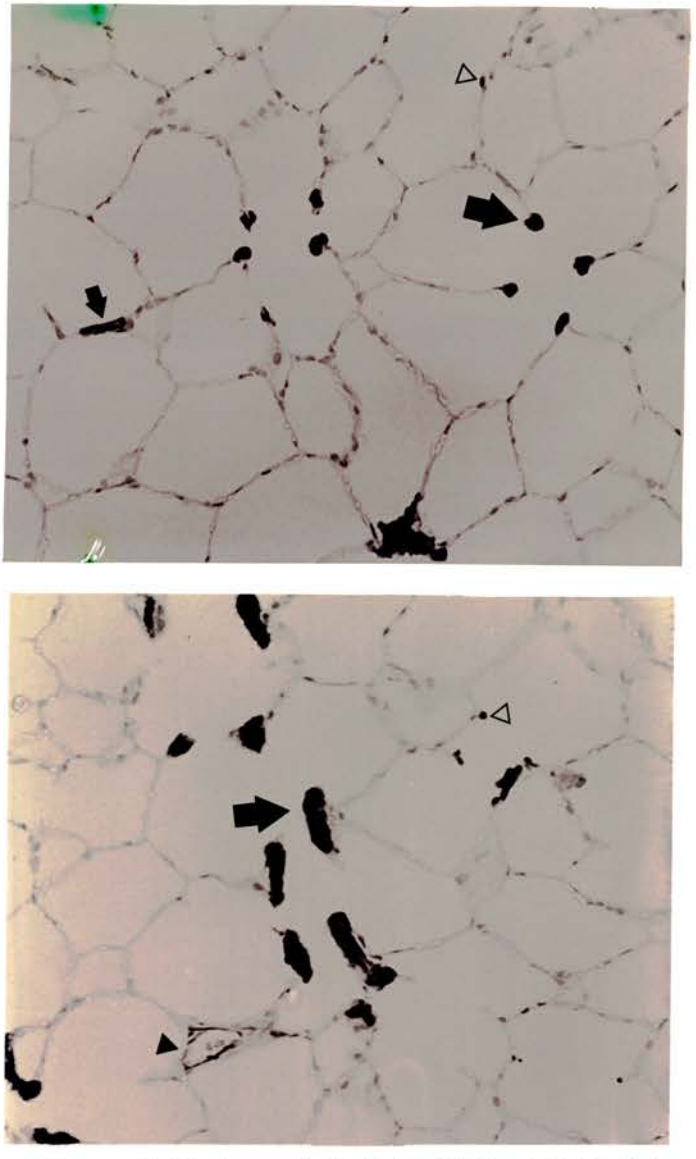


Figure 8.1 (top) & 8.2 (bottom). Both CON105, x200. Variation in thickness of alveolar septal tips is apparent between these two fields from the same section (large arrows). ASMA expression is also apparent in cells surrounding small venules at septal junctions (small arrows) and occasionally in septal cells (Δ). The latter cells are probably capillary pericytes.

8.3 STATISTICAL ANALYSIS

The shape of the distributions of V_{vt} and $V_{v'ASMA'}$ values for each sheep were examined visually. In addition statistical measures of skewness (G1) and goodness of fit to the normal distribution (Lilliefors test) were calculated. Where appropriate, arcsine transformations were used in an attempt to normalize distributions. Correlations between functional and morphometric indices were assessed using either the Spearman rank-order correlation coefficient (r_s) or the Kendall rank-order and partial rank-order correlation coefficient (T) as appropriate. In examining the relationship between contractile tissue and lung distensibility the amount of ASMA was expressed as the volume density of ASMA ($V_{v'ASMA'}$) and the total lung content of ASMA ($ASMA_{TOT}$). Statistical analyses were executed using commercially available software (Systat for Windows, v.5; Systat Inc., Evanston, IL, USA).

8.4 RESULTS

In the lungs from sheep seronegative for MVV, ASMA expression was located surrounding small vessels and airways and at the septal tips that abut the alveolar ducts. There was considerable variation, even within the same sections, in the thickness of alveolar septal tips and the level of ASMA expression of the same (Figure 8.1 & 8.2, facing page). Seropositive sheep in the earliest stages of pathology frequently presented little in the way of histopathological abnormality, however, parenchymal lymphoid interstitial infiltrates and follicular hyperplasia were often the earliest noted lesions. In these instances ASMA expression was frequently increased in the parenchyma immediately surrounding lymphoid infiltrates (Figure 8.3, below).

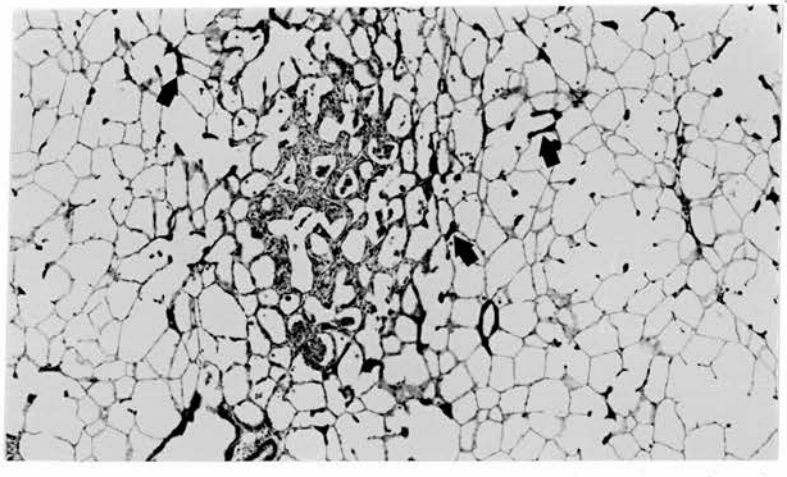


Figure 8.3. MVV032, x40 . Section from a sheep in the early stages of pathology. An early interstitial infiltrate is shown. There is an increase in ASMA expression in the parenchyma immediately surrounding the infiltrate (arrows). the expression decreases with distance from the infiltrate.

In other instances the increased ASMA expression both in the vicinity of the alveolar ducts and extending into alveolar septae was apparently unconnected with follicular development (Figure 8.4, below).

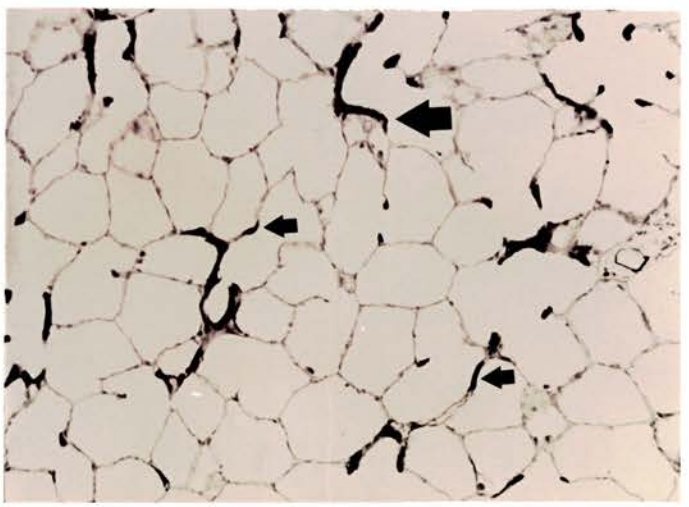


Figure 8.4. MVV007, x100. Section from a sheep with minimal pathology. There was no evidence of follicular development in the section, however increased ASMA expression is apparent in the vicinity of the alveolar ducts (large arrow) and also in alveolar septal walls (small arrows).

With more moderate pathology and a more generalized interstitial infiltrate there was an increased ASMA expression throughout the parenchyma, with the increased expression apparent in 'alveolar' areas but not extending into follicular structures (Figure 8.5, below).

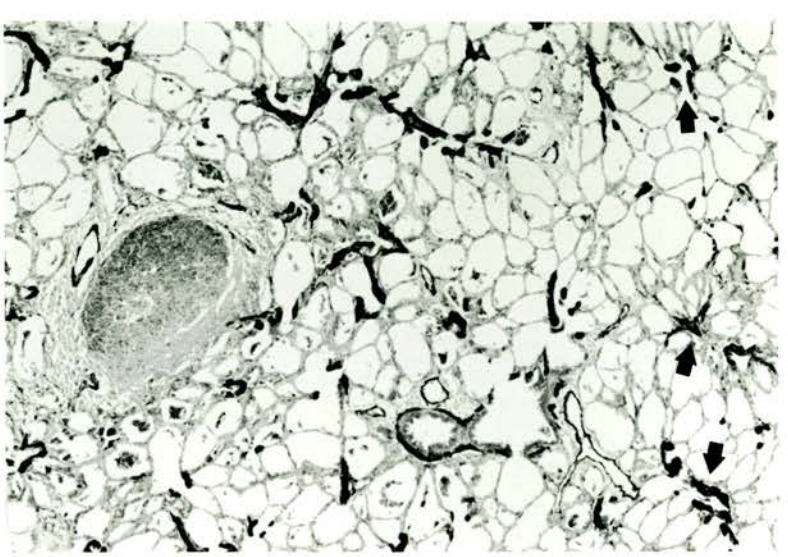


Figure 8.5 MVV068, x40 Variation in the width of alveolar ducts is apparent with some ducts appearing almost closed (arrows).

With advanced pathology, there was considerable variation in the extent of ASMA expression. The level of expression appeared to subjectively correlate with the degree of interstitial reaction, however in areas where interstitial infiltration was minimal there appeared to be many focal septal cells expressing ASMA (Figure 8.6, below) that were not apparent in sections from seronegative sheep or seropositive sheep with minimal to moderate pathology.

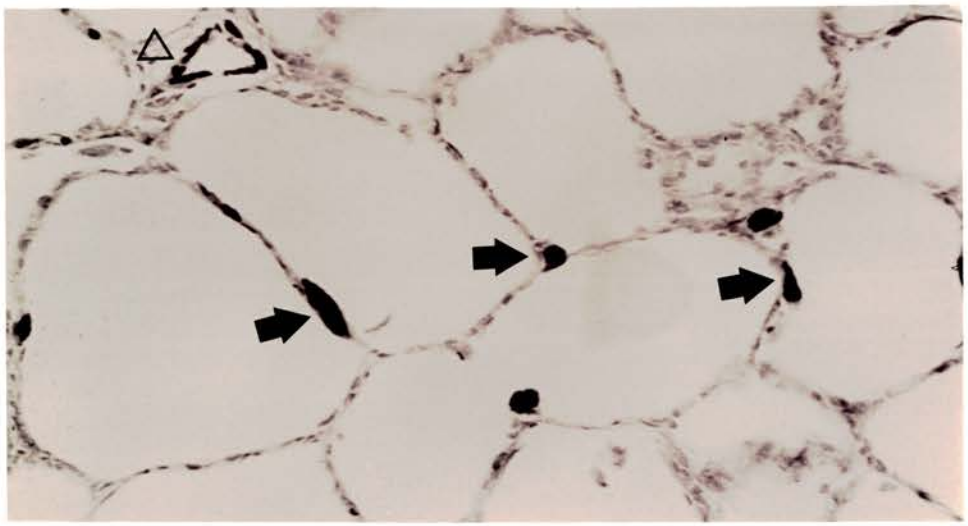


Figure 8.6. MVV068, x200. Section from a seropositive sheep with severe overall pathology. There is minimal interstitial reaction. There appears to be several individual septal cells expressing ASMA (arrows). These cells were less evident in seronegative sheep or sheep in the earlier stages of pathology. A small venule surrounded by ASMA expressing cells is apparent (Δ).

In areas of pronounced interstitial infiltration, ASMA expression was considerably increased with the distribution suggesting development from alveolar septal tips or individual foci within the septae themselves, rather than a diffuse increase in ASMA expression throughout all interstitial cells (Figure 8.7)(below) & (Figures 8.8 -8.9)(overleaf).

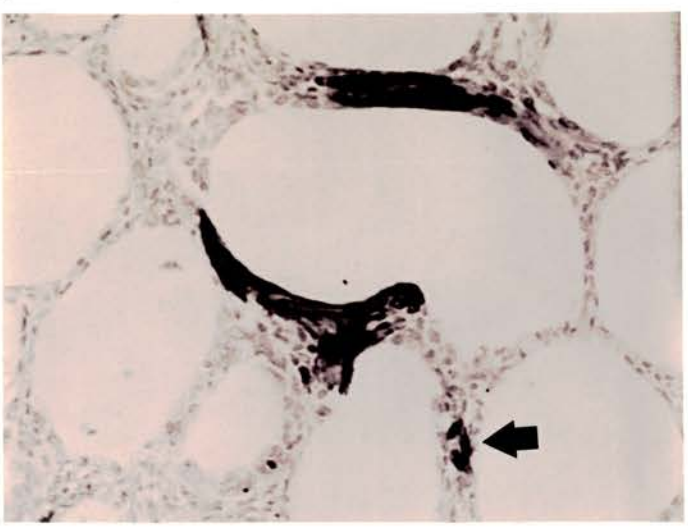


Figure 8.7. MVV068, x200. Increased ASMA expression in the interalveolar septae is evident. These areas may develop from septal foci of ASMA expressing cells (arrow).

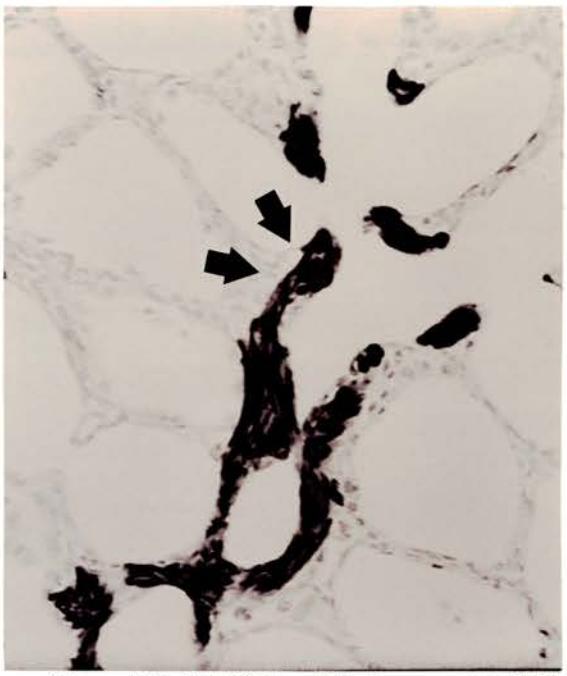


Figure 8.8. MVV068, x200. Increased ASMA expression appears contiguous with and may develop from septal tip cells (arrows).

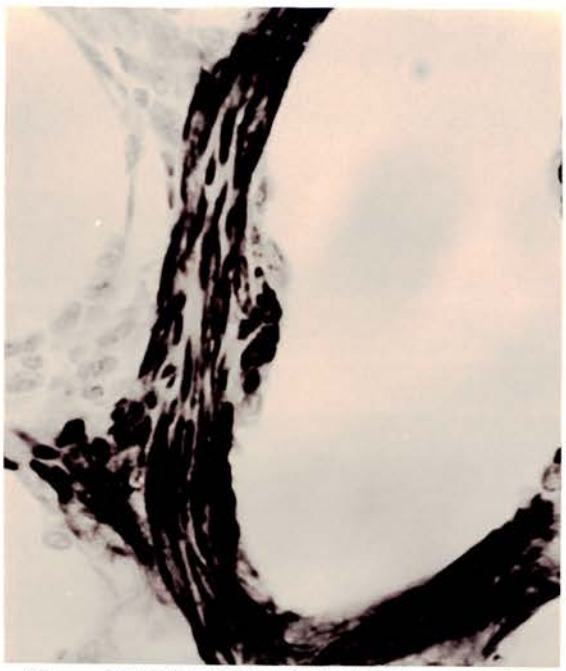


Figure 8.9. MVV068, x400. ASMA expressing cells in the interalveolar septae appear to be spindle shaped when longitudinally sectioned.

As LIP develops there is a concomitant increase in airspace size (Figure 8.10-8.11).

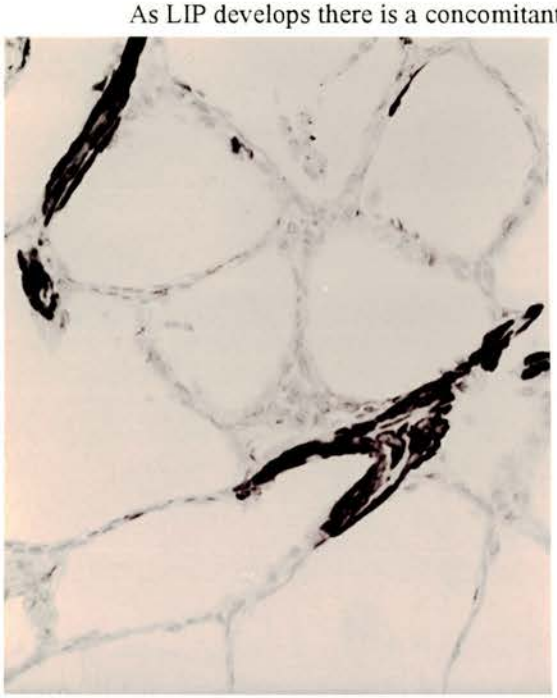


Figure 8.10 (above left) MVV068, x200. Increased interstitial cellularity and ASMA expression. Compare airspace size with Figure 8.11 (above right) which is a section from a control sheep (CON105, x200).

Summary statistics, including results of calculated measures of skewness (G1) and goodness of fit to normal distribution (Lilliefors) tests are given in appendices 8.1.1 - 8.1.3. The distributions of $V_{v'ASMA'}$ values were typically skewed to the right, appearing to approximate to the Poisson distribution, whereas those of V_{vt} values more often approximated to normal (Appendix 8.2). Arcsine transformations were unsuccessful in normalizing all the distributions as assessed using the Lilliefors test, therefore nonparametric statistical methods were used in analysis with median values being used as the relevant statistics of location.

Median $V_{v'ASMA'}$ values for individual sheep were positively correlated with median V_{vt} values for the same (r_s = 0.856; p<0.0005 (one-tailed))(Figure 8.12).

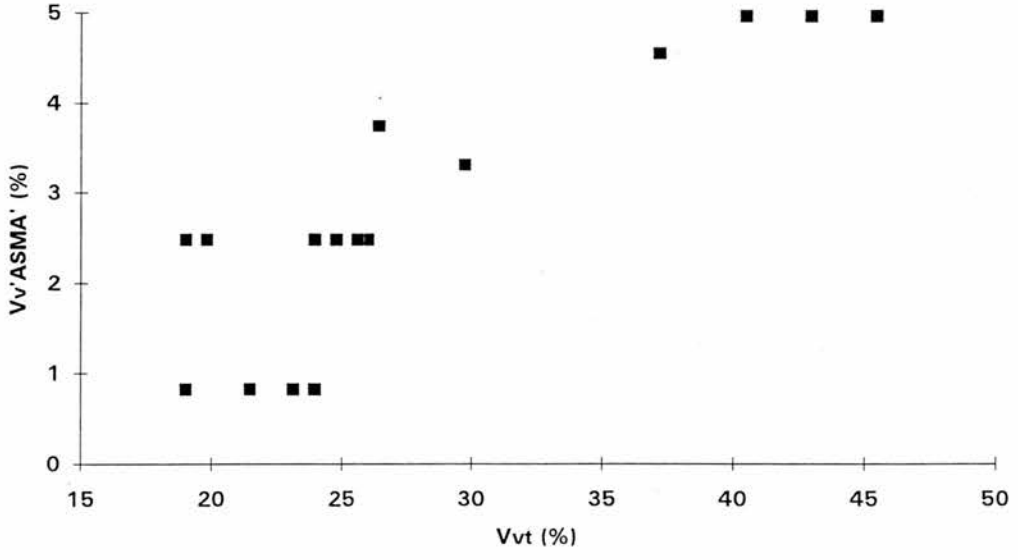


Figure 8.12 Relationship between the volume fractions of tissue (V_{vt}) and ASMA expressing tissue ($V_{v'ASMA'}$) in pulmonary parenchyma. The relationship is highly significant (r_s = 0.856; p<0.0005 (one-tailed)).

V_{vt} was negatively correlated with K and Cst (r_s = -0.615; p<0.005 and r_s = -0.683; P<0.0025 respectively (one-tailed)) and $V_{v'ASMA'}$ was negatively correlated with K and Cst (r_s = -0.614; p<0.005 and r_s = -0.504; P<0.025 respectively (one-tailed)). These associations are demonstrated in appendix 8.3. Kendall rank-order correlations, together with levels of significance between V_{vt}, $V_{v'ASMA'}$ and Cst and K are shown in table 8.1. The Kendall partial rank-order correlation coefficients are shown in table 8.2.

Variable	K	Cst (% Predicted)
V_{vt}	-0.444 (P<0.010)	-0.550 (P<0.005)
$V_{v'ASMA''}$	-0.465 (P<0.005)	-0.390 (P<0.025)

Table 8.1. Kendall T correlations between V_{vt}, $V_{v'ASMA'}$ and K and Cst. The levels of significance of the association between variables is shown in parenthesis.

Variable	K	Cst (% Predicted)
V_{vt} *	-0.218 (0.10<P<0.20)	-0.053 (P>0.25)
$V_{v'ASMA'}$ †	-0.157 (0.10<P<0.20)	-0.424 (P<0.01)

Table 8.2. Kendall partial rank-order correlation coefficients between morphometric and physiologic variables with $V_{v'ASMA'}$ (*) and V_{vt} (†) held constant. The levels of significance of the association between variables is shown in parenthesis.

Correlation results obtained when ASMA was expressed as the total lung content of ASMA ($ASMA_{TOT}$) were very similar to the above.

8.5 DISCUSSION

The contractile properties of lung parenchyma will largely depend on the quantity and isoform composition of the cellular protein actin within the constituent cells of the parenchyma. Mammals express at least six different actin genes in a tissue specific and species-independent manner: two non-muscle actins, two smooth muscle actins, cardiac muscle actin and skeletal muscle actin (Vanderkerckhove & Weber, 1978,1981). α-smooth muscle actin (ASMA) is the actin isoform typical of smooth muscle cells (Skalli *et al.*, 1987) and is expressed in normal lung parenchyma (Mitchell *et al.*, 1989,1991). Three populations of ASMA cells are described in normal human lung: (1) typical smooth muscle investing the large airways and blood vessels, (2) cells at the septal tips protruding into the alveolar ducts and (3) individual cells called pericytes located within the alveolar sac near the junctions of individual alveoli and in association with small blood vessels (Leslie *et al.*, 1990; Kapanci *et al.*, 1992). In lung injury, the quantity and distribution of ASMA expressing cells can change with the appearance of ASMA expressing cells, that resemble myofibroblasts, in the interstitium in bleomycin-induced pulmonary fibrosis in rats (Mitchell *et al.*, 1989; Vyalov *et al.*, 1993) and in idiopathic pulmonary fibrosis and hypersensitivity pneumonitis in humans (Leslie *et*

al., 1991). Outwith the lung ASMA is transiently expressed by myofibroblasts during experimental wound healing and appears to be instrumental in wound contraction (Darby *et al.*, 1990). As it is likely that ASMA expression is related to cellular contractile function this isoform was considered the most appropriate to quantify the amount of parenchymal contractile tissue in maedi and to therefore relate to the mechanical properties of the lungs.

The findings of this study demonstrate that all three populations of ASMA expressing cells described in normal human lung (Leslie *et al.*, 1990) were evident in the normal adult sheep lung. These studies also demonstrate that, in maedi, an increase in ASMA expression occurs both at the level of the alveolar ducts and also in the interstitium.

The progenitor of the interstitial ASMA expressing cells is unknown. One theory suggests that as post natal lung development proceeds, mesenchymal cells that previously expressed ASMA in the prenatal state cease expression of this protein in the normal adult lung. With lung injury one of the local effects of cellular mediators could be to stimulate the re-expression of this protein (Mitchell *et al.*, 1990). Certainly, cells within the normal alveolar septa, although not staining for ASMA, do appear to be laden with microfilaments and stain for anti-actin (Kapanci *et al*, 1974,1979), suggesting potential for contractile function. Further evidence that existing cells undergo a phenotypic modulation comes from the observations of Vyalov *et al.* (1993) that, in experimental pulmonary fibrosis in rats, increased ASMA expression occurs in interstitial desmin-containing cells that previously did not express ASMA. Another theory suggests that the capillary pericyte is the progenitor of these cells (Mitchell *et al.*, 1989) however Vyalov *et al.* (1993) considered this hypothesis unlikely given that pericytes are desmin negative whereas increased ASMA expression occurred in desmin-containing cells. Whatever the specific origin of these cells it is interesting to consider whether their appearance is purely a pathological consequence with no functional basis or whether, with their contractile capabilities, their appearance is an attempt at physiologic compensation.

Parenchymal lymphoid follicles and aggregates, a characteristic feature of maedi, appear to develop from initial sparse accumulations of perivascular and peribronchial lymphocytes (Cross *et al.*, 1975). The increase in ASMA expression adjacent to developing lymphoid follicular aggregates suggests a local association between these events, however the possibility that this might be a nonspecific feature of lymphoid follicular development throughout the body cannot be excluded. Are these contractile cells serving a predetermined functional role? One previous theory regarding the function of parenchymal contractile tissue in normal lung suggests a role in the local regulation of ventilation (Nadel *et al.*, 1964), with contraction shifting ventilation elsewhere. The increased

interstitial ASMA expression seen in bleomycin-fibrotic rat lungs (Low *et al.*, 1984) or in maedi could be interpreted as a local attempt to maintain ventilation-perfusion equality (Low *et al.*, 1984) and it is conceivable that even the increase in ASMA expression seen adjacent to early interstitial infiltrates in maedi is an attempt at diverting ventilation away from this area. Another possible function of these cells is suggested from their morphologic and phenotypic similarities to myofibroblasts involved in wound healing and contracture i.e. that these cells function to contract and reduce the size of nonfunctioning lung units (Adler *et al.*, 1989). Georgsson *et al* (1976) suggested that the increased contractile tissue seen in maedi was an attempt to compensate for loss of lung recoil brought about by the fragmentation and destruction of parenchymal elastic fibres seen in this condition. These theories suggest that in lung injury and disease the increased ASMA expression is spatially related to the pathology, however increased expression can also develop in areas apparently devoid of pathology. This apparent contradiction could be explained if the factor(s) inducing ASMA expression have a more diffuse distribution than is suggested by the photomicrographs of early follicle development. The alternative suggestion is that the increased ASMA expression has no *a priori* function, that its appearance is an idiosyncrasy of the disease pathogenesis.

What are the likely stimuli for ASMA expression in maedi? The growth, proliferation and differentiation of lung mesenchymal cells is governed by a complex linking of physical and chemical influences. Evidence supporting the role of the extracellular matrix (ECM) in this regulation is cited by Adler *et al.* (1989). Both biochemical and physical aspects of the ECM composition are thought to interplay with the cells therein. Bissel *et al* (1982) propose a model of 'dynamic reciprocity' whereby the ECM can modify cytoskeletal elements, modifications which could lead to a reciprocal influence on the production and decay of ECM components. The close proximity and physical linking of ECM components to cells will lead to forces being applied directly to the cytoskeleton. Ingber & Jamieson (1985) reason that the cells act as 'tenegrity structures' that accomodate and translate these mechanical events according to the stress applied. Indeed, human lung fibroblasts *in vitro,* proliferate and realign in response to cyclic deformation, an event thought to be mediated by autocrine growth factors (Bishop *et al* 1993). The increased expression of ASMA in maedi may represent cytoskeletal reorganization in response to changes in the biochemical or physical composition of the ECM. Alternatively, a specific disease-related stimulus might be responsible for the increase in ASMA expression. Whatever the initial stimulus, the final pathway is likely to involve the action of a chemical mediator or cytokine, and it is interesting to consider, in the light of maedi pathogenesis, which of the known cytokines might be involved. The main target cells for infection by the MVV are the mononuclear phagocytes, the monocytes and macrophages. In the lung, the number of MVV-infected alveolar macrophages (AMs) increases with severity of LIP (Brodie *et al.*, 1992) suggesting that these cells play a pivotal

role in pathogenesis. In addition, although only a small proportion of AMs might be infected with MVV, a more widespread activation of AMs occurs (Cordier *et al.*, 1990), and these activated cells are likely to increase their production of secretory products, perhaps to levels of pathologic significance. Transforming growth factor-β (TGF-β) is one of the known cytokine growth factors secreted by AMs, and this peptide exerts control over the growth, differentiation, and function of mesenchymal cells, including muscle cells (Roberts & Sporn, 1989). In particular, recent studies have demonstrated that this peptide induces the *in vitro* expression of ASMA by rat lung mesenchymal cells (Mitchell *et al.*, 1993) through regulation at the translational or post-translational level. The role of this cytokine in maedi is speculative, however, further *in vivo* studies relating changes in TGF-β and mRNA expression to actin isoform expression should furnish objective data regarding its role in this condition.

Other cytokines may be involved in the modulation of cellular phenotypes towards ASMA expression in the lung. Platelet-derived growth factor (PDGF) is a potent mitogen, rendering cells competent to proceed around the cell cycle and divide (Sappino *et al.*, 1990; Kelley 1990; Shaw 1991). It is strongly implicated in fibrotic lung disease (Shaw, 1991) and is thought to play an essential role in the early stages of tissue repair. Influence on ASMA expression in the lung may relate to control over TGF-β activity in that cells that cannot both secrete and respond to PDGF will not respond to TGF-β (Kelley, 1990). Other cytokines likely to contribute to the control of fibroblast differentiation include: (a) fibroblast growth factor (FGF), a potent inducer of neovascularisation produced by macrophages and endothelial cells (Sappino *et al.*, 1990; Kelley 1990), (b) granulocyte-macrophage colony stimulating factor (GM-CSF), which can be shown to induce the accumulation of ASMA expressing fibroblasts following subcutaneous perfusion in rats (Rubbia-Brandt *et al.*, 1991) and (c) interferon-γ, which can reduce ASMA expression in cultured smooth muscle cells (Sappino *et al.*, 1990). The possible role of ECM components such as proteoglycans, heparin and fibronectin in influencing phenotypic differentiation should also be acknowledged (Sappino *et al.*, 1990).

The quantity of parenchymal contractile tissue is increased in several human lung and cardiovascular diseases (Adler *et al.*, 1989; Kapanci *et al* 1990; Leslie *et al.*, 1991). Although the functional significance of this element is unknown, intuitive supposition would suggest potential effects on lung mechanics at least at the local level, and further suggests that specific therapeutic intervention aimed at this element might be capable of alleviating some of the symptoms of these conditions.

One objective was to demonstrate whether a relationship exists between lung distensibility and the quantity of parenchymal contractile tissue in maedi. Initial scrutiny of the data would suggest that this is the case, however although correlations exist between the morphometric and functional indices, partial correlation coefficients indicate that the former are strongly interdependent. If V_{vt} is considered representative of the interstitial reaction one can conclude that without an interstitial reaction, no relationship would exist between $V_{v'ASMA'}$ and K. Given the strong correlation between $V_{v'ASMA'}$ and V_{vt} and subjective observations of the relationship between these variables it is not surprising that it is not possible to statistically separate the influence of one from the other. The analogous experimental control would be to select a range of animals with wide variation in $V_{v'ASMA'}$ but not V_{vt}, an onerous requirement that would be virtually impossible to satisfy. The significant partial correlation coefficient between $V_{v'ASMA'}$ and Cst is misleading as it suggests a relationship independent of the interstitial reaction, however these variables themselves are not necessarily independent as both will be influenced by lung volume.

The positive relationship between $V_{v'ASMA'}$ and K should not be interpreted as a causal link, other factors associated with the interstitial reaction eg the composition and structure of the interstitial matrix, the quantity and distribution of contractile tissue surrounding larger airways and blood vessels, the pulmonary capillary blood volume or the composition of lung surfactant might play equally important roles. However, initial studies suggested $V_{v'ASMA'}$ was a likely candidate for effecting a change in the mechanical properties of the lungs. These results do not disprove such a link. That a relationship, albeit clouded by the possible influence of parameters beyond measurement or control, can be demonstrated is interesting in that only the quantity of ASMA is considered in this study, whereas in the dynamic situation, both the quantity and functional tone will presumably influence mechanical properties. Thus further studies are required to relate the quantity of parenchymal contractile tissue to the *in vivo* functional effects of its contraction.

8.6 SUMMARY

The distribution and morphometric quantitation of α-smooth muscle actin (ASMA) in lung parenchyma was determined in 3 normal sheep lungs and in 15 lungs from sheep seropositive for MVV. The relationship between the volume density of ASMA in lung parenchyma ($V_{v'ASMA'}$) and static lung compliance (Cst) and lung distensibility (K) was examined.

In control lungs, ASMA was expressed by typical smooth muscle cells surrounding airways and blood vessels, by cells at the alveolar septal tips protruding into the alveolar ducts and rarely by individual cells within septae. In MVV-seropositive sheep with minimal histopathology increased

ASMA expression occurred in association with early interstitial infiltrates and was located both at septal tips and within septae. With more severe pathology, ASMA expressing cells became organised into bundles within obviously thickened septae and septal tips.

In maedi, $V_{v'ASMA'}$ is negatively correlated with K and Cst (r_s = -0.614; p<0.005 and r_s = -0.504; P<0.025 respectively). However, partial correlation coefficients indicate that $V_{v'ASMA'}$ and lung parenchymal tissue density (V_{vt}) are strongly interdependent making it difficult to interpret the link between $V_{v'ASMA'}$ and K. The above findings make a positive contribution towards the hypothesis that lung parenchymal contractile tissue is capable of influencing overall lung mechanics.

CHAPTER 9

QUANTIFYING THE INFLUENCE OF SMOOTH MUSCLE HYPERPLASIA ON PERIPHERAL AIRWAY DYNAMICS IN MAEDI

9.1 INTRODUCTION

Lung functional disturbances associated with MVV infection include reduction in static lung compliance (Cst) and lung distensibility (K) and an increase in lung elastic recoil (Chapters 4 & 7). These changes in lung elastic properties are associated with a reduction in effective alveolar lung volume ($V_{A,eff}$). Structure-function analysis reveals that there is an increase in airspace size in maedi therefore the density of surface forces are unlikely to account for the reduction in lung distensibility and it is suggested that tissue forces must play a major role in limiting lung distensibility in maedi.

Tissue forces relate to the presence of noncellular fibrous elements in the lung and to the presence of contractile elements associated with conducting airways and blood vessels, and with the peripheral airways i.e. the respiratory bronchioles and alveolar ducts. In maedi there is between a two- and six-fold increase in the quantity of parenchymal contractile tissue at the level of the peripheral airways, as assessed by the volume density of the actin isoform α-smooth muscle actin ($V_{v'ASMA'}$)(Chapter 8).

Published evidence demonstrates that in paralyzed, artificially ventilated cats, constriction of these peripheral airways can cause a reduction in pulmonary compliance, an expulsion of air from the lungs and an increase in lung elastic recoil (Nadel *et al.*, 1964; Colebatch *et al.*, 1966). Colebatch & Mitchell (1971) demonstrated similar changes in isolated liquid-filled lungs thus indicating that surface forces cannot account for this phenomenom. Furthermore, these latter authors observed that the magnitude of constriction was qualitatively related to the amount of alveolar duct muscle in cat lungs. That a similar relationship might hold for sheep is supported by the observation that barium sulphate embolism (Halmagyi & Colebatch, 1961) and parenteral administration of histamine (Hutchison *et al.*, 1982) cause similar functional changes in sheep as do occur in cats, although the exact mechanism has not been demonstrated by direct means. Further support relates to the observed correlation between parenchymal contractile tissue and lung distensibility in maedi (Chapter 8).

To establish whether, in sheep, lung elastic properties are influenced by the contractile tissue at the level of the peripheral airways would require the demonstration that contraction or relaxation of this element was associated with changes in these properties. To further establish whether the relative quantity of contractile tissue at the level of the peripheral airways is a significant factor in determining the degree of change in the above functional parameters would require the demonstration of a relationship between the degree of functional change produced by a given stimulus and the quantity of contractile tissue. Both questions are relevant to establishing whether, and to what extent the increased quantity of parenchymal contractile tissue previously identified in maedi is of functional significance.

The following describes an investigation into determining the relationship between the quantity and functional tone of parenchymal contractile tissue and lung elastic properties in control sheep and sheep with maedi. Histamine was selected as an appropriate agonist of peripheral airway contractile tissue. Histamine (β-aminoethylimidazole) is an amine that occurs as a decomposition product of histidine and is prepared synthetically from it. It is widely distributed in an inactive form in the body, particularly in cytoplasmic granules of mast cells and basophils. The actions of histamine are mediated through two distinct receptors (H_1 & H_2). Activities mediated through H_1 receptors include smooth muscle contraction (White & Kaliner, 1991). Administration of histamine to sheep by intravenous infusion causes a reduction in dynamic compliance and an inconsistent increase in lung resistance and functional residual capacity (Hutchison *et al.*, 1982). The former change is believed to be brought about by smooth muscle contraction in the peripheral airways (Hutchison *et al.*, 1982). If the hypothesis linking the quantity of contractile tissue to lung elastic properties is valid, then the dose-response relationship should relate to the quantity of contractile tissue. Clenbuterol [benzyl alcohol, 4-amino-α-(t-butylamino) methyl-3,5-dichloro], a β-2 sympathetic agonist, was chosen as a putative relaxant of peripheral airway contractile tissue. Clenbuterol is a synthetic β-adrenergic agonist used in veterinary medicine as a bronchodilator, as a uterine muscle relaxant and as a repartitioning agent (Brockway *et al.*, 1987). Intravenous administration of β-agonists are capable of altering elastic recoil in human lungs and this change is believed to be associated with relaxation of alveolar duct smooth muscle (DeTroyer *et al.*, 1978) Again, if the lung elastic properties are determined by the quantity of the parenchymal contractile tissue, then the dose response of changes in these properties should relate to the latter.

9.2 MATERIALS AND METHODS

9.2.1 Animals

14 adult Texel ewes were used in this study. 8 were seropositive for MVV and 6 seronegative for MVV on the basis of the agar gel immunodiffusion test (Winward *et al.*, 1979). All sheep were managed under similar conditions. Details of the sheep used in this study are given in appendix 9.1. Control sheep were accepted as normal on the basis of a thorough clinical examination and the absence of signigficant haematological abnormalities.

9.2.2 Animal preparation

Food was withheld for 12 hours prior to anaesthesia. Immediately prior to anaesthesia the area over the left jugular vein was clipped and an intravenous catheter (14G,57mm)(Quik-Cath, Travenol Laboratories Inc., IL, USA) sutured in place.

9.2.3 Anaesthesia

Anaesthesia was induced by intravenous injection of a single bolus of thiopentone sodium (Intraval Sodium; Rhône Mérieux Ltd.) at a dose rate of 20 mg/kg bodyweight as previously described (Chapter 2; section 2.2.2). Anaesthesia was maintained, via the jugular catheter, with an infusion of 0.5% thiopentone sodium in saline (Aqupharm No 1; Animalcare Ltd., UK) adjusted to maintain a dose rate of 0.20-0.25 mg/kg/min. The maintenance infusion was heparinized by the addition of lithium heparin (Heparin Injection BP; Leo Laboratories Ltd., Bucks, UK) to give a final concentration of 2 IU/ml.

9.2.4 Instrumentation

Instrumentation of the anaesthetized sheep was as previously described (Chapter 2; section 2.2.2) excepting that the length of the latex oesophageal balloon was 7cm (diameter 1.5cm, thickness 0.05mm) and 1ml of air was added to the evacuated balloon prior to measurements commencing. Positioning of the balloon in the caudal third of the thoracic oesophagus and the pressure-volume characteristics of the balloon catheter assembly were validated as previously described (Chapter 2.2.2).

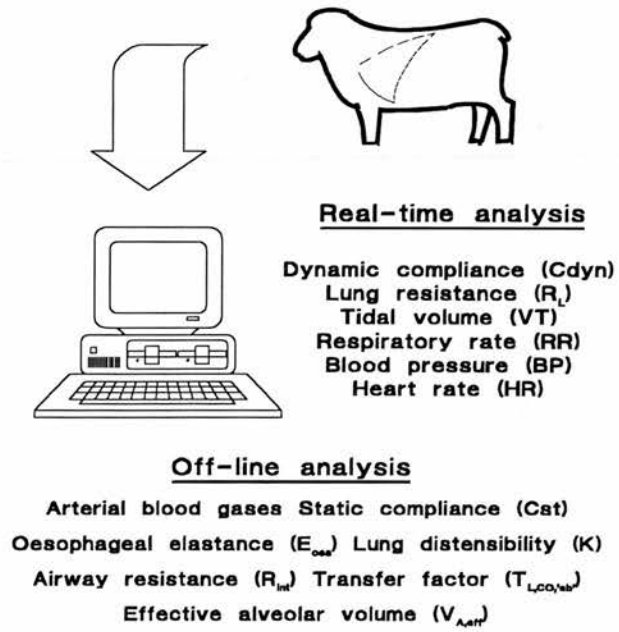


Figure 9.1 Instrumentation allows respiratory flow (V), transpulmonary pressure (Ptp) and systemic blood pressure (BP) to be continuously monitored. This data is acquired using an ADC board in an Apple Macintosh IIsi computer. The variables derived from real-time analysis and off-line analysis are indicated.

In addition to the routine instrumentation used in static determinations of lung volume, lung compliance and distensibility and of transfer factor, further instrumentation to enable the real-time analysis of systemic blood pressure and lung function was required. Figure 9.1 (above) illustrates the instrumentation used and summarises the analysis procedures described in the following sections.

9.2.4.1 Arterial blood sampling and blood pressure recording

The middle auricular artery was selected as appropriate for catheterization. Techniques are described and illustrated for sampling auricular arteries in the bovine (Riley & Thompson, 1978; Fisher *et al.*, 1980; Trim, 1980; Oakley *et al.*, 1980; Muller & Goetze, 1987) and a similar technique was used in the sheep. The dorsal surface of the ear was shaved and prepared using appropriate antiseptic techniques. Once the ear is shaved the middle auricular artery is easily visualized and palpated as it pursues its course along the dorsal convex ear surface from base to apex. A small stab incision with a scalpel blade (No. 15; Swann Morton Ltd., Sheffield, UK) was made immediately to one side of the artery. The position of the artery was stabilized using fingertip pressure and an over-the-needle catheter (20G,32mm)(Biovalve; Vygon Ltd., Ecouen, France) was advanced through the stab incision and into the arterial lumen. The catheter was immediately flushed with heparinized

saline and taped or stitched to the ear. Saline filled fluid lines were used to connect the catheter to a suitable blood pressure transducer (Type 4-327-I; TransAmerica Delaval Medical Products, USA) which was in turn connected to an appropriate component transducer preamplifier (Devices 3559/3550) of an 8-channel chart recorder (Devices MI9; Devices Ltd, Herts, U.K.). The catheter was periodically flushed with heparinized saline (2 IU/ml) to maintain patency and absence of air bubbles in the fluid lines was carefully checked both prior to and during procedures. The transducer was zero referenced at the point of the shoulder.

Arterial blood samples were obtained from the auricular artery catheter using disposable 2ml heparinised syringes (2ml Monovette LH; Sarstedt, Nümbrecht, W. Germany). An arterial blood gas analyser (Ciba Corning 238; Ciba Corning Diagnostics Ltd., Essex, UK) was used to make the appropriate blood gas and acid-base determinations.

9.2.4.2 Real-time analysis

It was considered appropriate to invest time in developing a system that would allow on-line analysis of transpulmonary pressure, respiratory flow and blood pressure data such that the functional response to given pharmacological mediators could be continuously monitored during the period of anaesthesia. An additional requirement of the system was the capability to store raw data as well as computed parameters for further analysis. A description of the system follows in two parts, the first concerning the data acquisition aspect and the second the analysis aspect.

(a) Data acquisition: Conditioned analogue signals (±1V) from the pressure and flow transducers (CS9; Mercury Electronics, Glasgow, UK) and from the blood pressure transducer preamplifier (± 5V) were connected to a 12-bit ADC data acquisition board (Lab-NB; National Instruments, Austin, TX, USA) within an Apple Macintosh IIsi computer (9Mb RAM, 80Mb HD). The data acquisition functions of the data acquisition board were controlled by an instrumentation software system (LabView 2; National Instruments, Austin, TX, USA)(Appendix 9.2). Analogue input channels were sampled at 100Hz/channel. Double-buffered data acquisition was configured such that on-line analysis could be completed without interrupting the flow of data into a large circular buffer.

(b) Data analysis: Using LabView 2 software, a virtual instrument (VI) was constructed to analyse transpulmonary pressure and respiratory flow data in such a way that the following parameters were calculated:- Dynamic compliance (C_{dyn}), total pulmonary resistance (R_L) and respiratory rate (RR). Definitions of these parameters and the methods of derivation are shown in Appendix 9.3. In addition the blood pressure trace was analysed to yield the heart rate (HR) and mean blood pressure

(BP). These parameters were computed at 20 second intervals and displayed on the 'front panel' of the VI as separate strip charts or digital indicators. In addition to the real-time graphic display of computed parameters, a facility was incorporated to allow visualization and the option to save raw data traces as well as their associated computed measurements.

The 'front panel' of the VI used to graphically display real-time data is shown in figure 9.2 overleaf. An example of transpulmonary pressure, respiratory flow and blood pressure data acquired using this VI is shown on the subsequent page (Figure 9.3).

9.2.5 Measurement protocols for off-line analysis

9.2.5.1 Static lung mechanics, lung volumes and transfer factor

The protocol for the collection of static lung pressure-volume data was as previously described (Chapter 2; section 2.2.3). Static compliance (Cst) was estimated by determining the slope of the line of best fit over the linear portion of the curve and lung distensibility (K) and other indices of exponential curve fitting were derived as previously described (Chapter 3; section 3.2.5). Static lung volume ($V_{A,eff}$) and single-breath transfer factor for carbon monoxide ($T_{L,CO,'sb'}$) measurements were obtained as previously described (Chapter 2; section 2.2.3). Respective static lung mechanics, lung volumes and transfer factor measurements were calculated on the basis of data collected from single lung inflation-deflation cycles.

Data was acquired using the previously described software directed data acquisition package and the necessary data points selected at a later time point using a graphic display of data and interactive cursor measurement VI.

Water and mercury manometers were used to calibrate respective transpulmonary and blood pressure transducers. Calibration of the flow transducer-pneumotachograph assembly was achieved with a flow meter (Rotameter 2000; G.E.C. Elliot, Process Instruments Ltd., Croydon,U.K.) and pressure and flow measuring devices were phase matched to a frequency of 10Hz using techniques similar to those decribed by Macklem (1974). A calibrated syringe was used to assess accuracy and linearity of the flow recording system in the measurement of volumes.

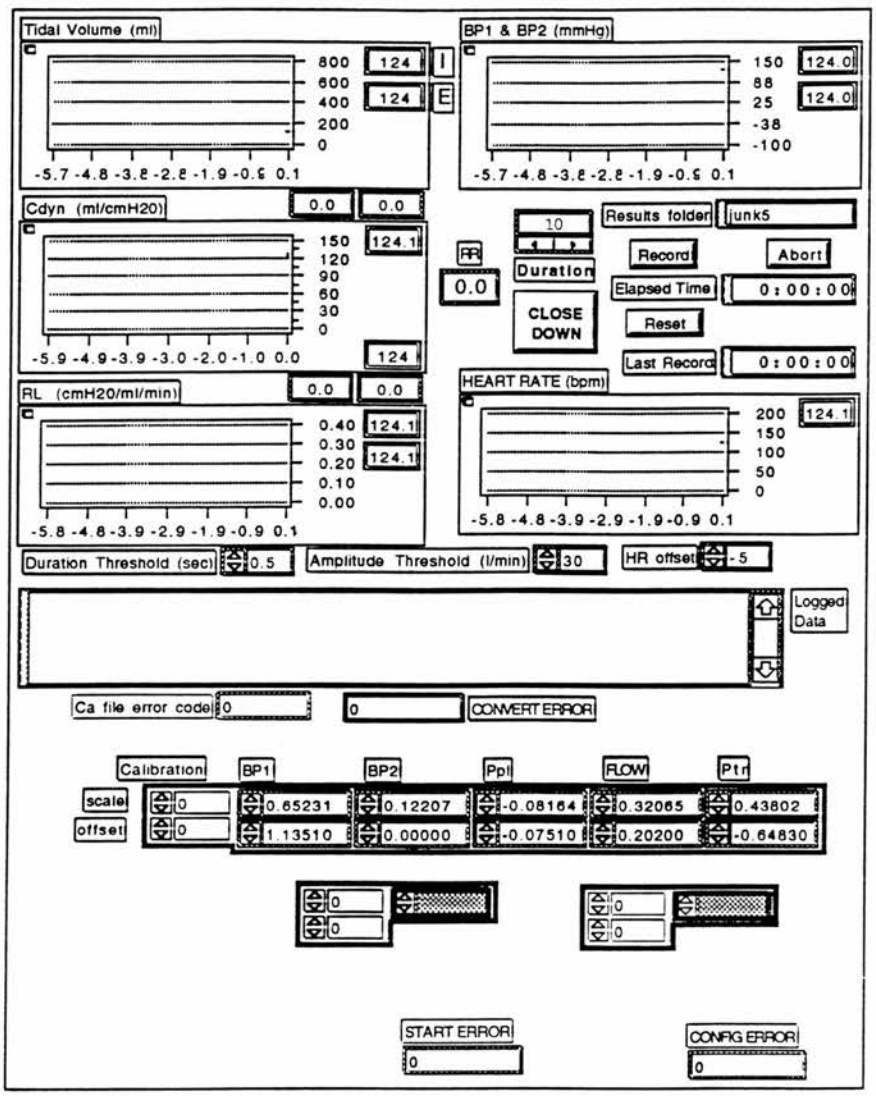


Figure 9.2 Front panel of the VI used to graphically display real-time data on the colour monitor. Individual strip charts and digital indicators are self-explanatory, these being updated every 20 seconds. Calibration data is entered prior to running this VI. The VI runs continually and provides an instant display of respiratory and cardiovascular data. Data and computed parameters are only logged to disk when the record button is pressed. An example of the raw data is depicted overleaf.

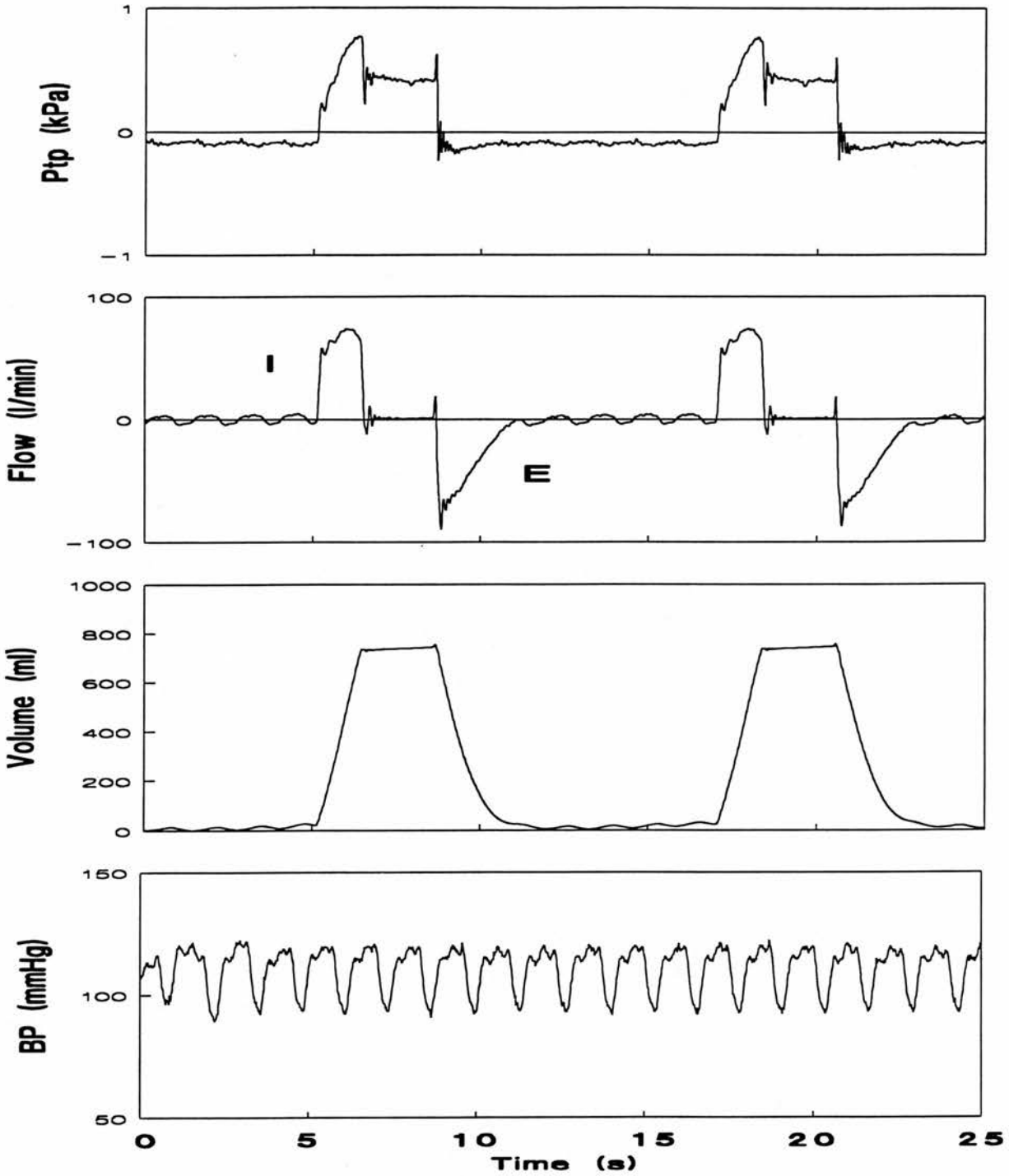


Figure 9.3 Examples of pressure, flow, volume and blood pressure data sampled by the ADC board. This data was logged for further analysis. In the flow trace, I and E indicate the inspiratory and expiratory breaths respectively.

9.2.5.2 Oesophageal elastance

Oesophageal elastance (E_{oes}) was calculated using a modification of the method described by Senterre and Geubelle (1970). Transpulmonary pressures (Ptp) at the end-expiratory pause were measured with different balloon volumes during mechanical ventilation (Figure 9.4). The volume changes of 0.5 ml were injected by syringe via a three-way tap. A volume of 2.5 ml was injected. The maximal balloon volume (3.5ml) was within the range of high compliance of the balloon-catheter system i.e. the transmural pressure of the balloon outside the oesophagus was 0 kPa. The slope of the relationship between the volume changes and the variations in Ptp at the end expiratory level was calculated by least squares linear regression and the result expressed in ml/kPa. Results were also expressed as the specific elastance of the oesophagus (sE_{oes}) i.e. the pressure variation per unit volume change per unit length of balloon (kPa/ml/cm).

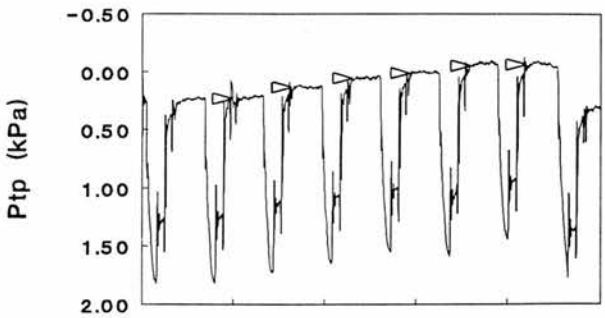


Figure 9.4. Transpulmonary pressure recording. The end-expiratory pressures (arrows) were recorded and plotted against volume injected.

9.2.5.3 Airway resistance by the interrupter technique

The interrupter technique as described by Gottfried *et al.* (1984) was used to measure airway resistance. Airway opening pressure (Pao) was measured by turning a three-way tap in the oesophageal balloon catheter assembly pressure line to atmosphere. During normal mechanical ventilation, the airway opening was occluded for several seconds at end inspiration. The airway

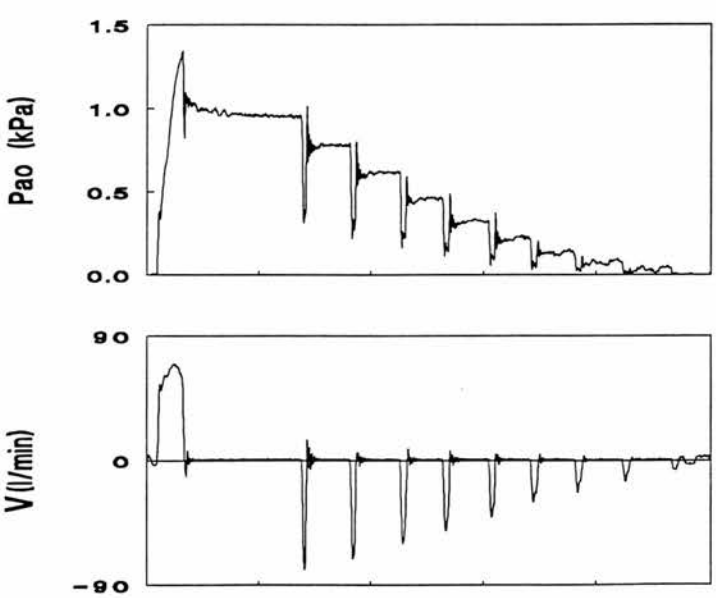


Figure 9.5 Recordings of airway opening pressure (Pao) (upper graph) and airflow (V) (lower graph) during the estimation of airway resistance (R_{int}) by the interrupter technique.

was rapidly reopened, and a series of interruptions performed by brief manual occlusions of the airway opening during the relaxed expiration (Figure 9.5). Airway resistance by the interrupter method (R_{int}) was obtained by dividing Pao immediately prior to interruption by the volume flow rate of expired air just prior to interruption. The method of backward extrapolation was used to estimate Pao immediately prior to interruption (Jackson *et al.*, 1974)(Appendix 9.4).

9.2.6 Experimental protocol

Where both histamine and clenbuterol were administered to sheep, these administrations were completed on different days with at least a one-month interval between procedures.

Prior to administration of histamine or clenbuterol the sheep were prepared as described and real-time functional data continuously monitored to ensure that stable respiratory and blood pressure baselines were achieved.

9.2.6.1 Histamine administration

The dose response to intravenous histamine was assessed using a similar protocol to that described by Hutchison *et al.* (1982). The dose response of 13 sheep (5 seronegative and 8 seropositive for MVV (Appendix 9.1)) was determined. Histamine was delivered by intravenous infusion using a peristaltic pump (P-1; Pharmacia Fine Chemicals, Sweden) set to a constant rate. Histamine solution was prepared fresh each day by dissolving histamine diphosphate (BDH Laboratory Supplies, Poole, UK) in saline (Aqupharm No.1; Animalcare Ltd., York, UK). Separate histamine solutions were prepared for each sheep such that, at the known pump infusion rate, dose rates of 0.01, 0.10, 1.0 and 3.0 μg histamine/kg/min were available.

Baseline data was collected prior to the administration of histamine. This data included real-time functional measurements (C_{dyn}, R_L, V_T, RR, HR, & BP) as well as data collected for the off-line analysis of arterial blood gases, Cst, *K*, $V_{A,eff}$, $T_{L,CO,'sb'}$, E_{oes}, and R_{int}.

Following baseline measurements successively more concentrated histamine solutions were administered for 5 minute periods until C_{dyn} was reduced to less than 65% of the baseline level and/or R_L was increased more than twofold, this criterion being defined as an acceptable end point response to histamine infusion. Data for off-line analysis was collected at the end of each infusion

period. The order of collection of this data was (1) arterial blood sampling, (2) E_{oes}, (3) R_{int}, (4) $V_{A,eff}$ and $T_{L,CO,'sb'}$ and (5) *K*. Sheep were carefully monitored for any untoward systemic manifestations of the histamine infusion.

9.2.6.2 Clenbuterol administration

The response of 9 sheep (2 seronegative and 7 seropositive for MVV (Appendix 9.1)) to intravenous injection of clenbuterol was determined. Following establishment and recording of baseline conditions an intravenous injection of a 30μg/ml solution of clenbuterol hydrochloride (Ventipulmin; Boehringer Ingelheim, Berks, UK) was administered (0.8μg/kg) via the jugular catheter. Repeated arterial blood sampling and real-time analyses were carried out during the 15 minutes immediately following injection. Subsequent to this measurements were made for off-line analyses.

9.2.6.3 Data Reduction

Data were expressed as percent of baseline levels preceding infusions of histamine or injection of clenbuterol. The dose of histamine that would be required to reduce C_{dyn} to 65% of baseline values ($ED_{65}C_{dyn}$) or increase R_L to 200% of baseline ($ED_{200}R_L$) was calculated by linear interpolation between the penultimate and final doses of histamine administered.

9.2.7 Morphometric Analysis

Nine sheep (2 seronegative and 7 seropositive for MVV (Appendix 9.1)) were euthanazed following drug administration protocols and their lungs obtained for morphometric analysis. Necropsy and tissue fixation procedures were as previously described (Chapter 5; section 5.2). Tissue block sampling, immunostaining for α-smooth muscle actin (ASMA) and morphometric analysis of tissue sections were completed as described in chapter 8.

9.3 STATISTICAL ANALYSIS

Non-parametric statistical tests were used in analysis. In comparisons of independent samples, the Mann-Whitney test was employed and in the case of paired samples the Wilcoxon signed ranks test was used. Correlations between variables were assessed using Spearman (r_s) rank-order correlation coefficients.

9.4 RESULTS

9.4.1 Histamine infusion

Summary statistics regarding baseline C_{dyn}, R_L, R_{int}, E_{oes}, sE_{oes}, BP and arterial blood gas and acid-base values are shown in table 9.1 below. These measurements were made in seronegative sheep.

	BP	C_{dyn}	R_L	E_{oes}	sE_{oes}	R_{int}	
Statistic	(mmHg)	l/kPa	kPa/l/s	kPa/ml	kPa/ml/cm	kPa/l/s	
Mean	127	0.872	0.551	0.100	0.702	1.433	
S.D.	9.4	0.533	0.176	0.030	0.207	0.546	
Mimimum	116	0.366	0.330	0.051	0.359	0.978	
Maximum	142	1.778	0.731	0.127	0.891	2.064	
Median	127	0.728	0.509	0.105	0.737	1.151	
	pH	$PaCO_2$	PaO_2	HCO_3	tCO_2	BEvt	O2sat
Statistic	-	kPa	kPa	mmol/l	mmol/l	-	%
Mean	7.52	4.67	14.80	29.6	28.1	5.6	97.9
S.D.	0.051	0.550	3.507	3.16	3.59	3.46	1.35
Mimimum	7.47	4.27	9.86	24.7	22.8	0.2	95.6
Maximum	7.60	5.33	18.26	32.7	31.1	8.9	98.9
Median	7.52	4.27	13.86	30.4	29.7	6.6	98.3

Table 9.1 Summary statistics for baseline values of variables reflecting respiratory and cardiovascular function.

Examples of typical changes in real-time variables (HR, BP, C_{dyn}, & R_L) with successive histamine infusions are shown in figure 9.6. Both real-time data and the results of off-line data analysis are given in appendix 9.5 & 9.6.

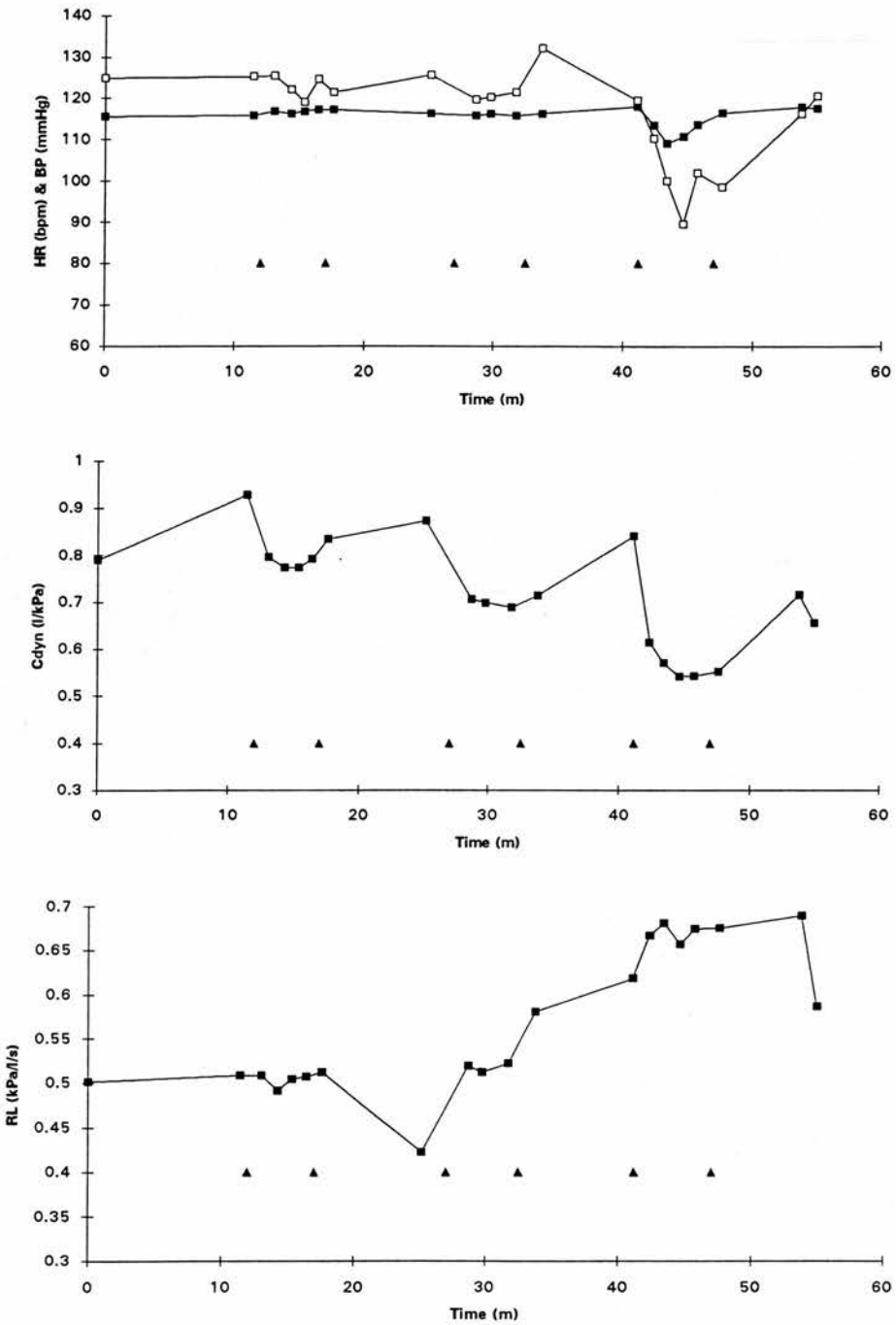


Figure 9.6 Real-time data during successive 5 min histamine infusions (0.01, 0.1 & 1.0 μg/kg/min)(CON094). The start and end points for infusions are indicated (▲). Uppermost graph, □ - HR (bpm), ■ - BP (mmHg).

The results of Wilcoxon signed ranks tests are given in table 9.2.1 (overleaf).

	CONTROL (n=5)		MAEDI (n=8)	
Variable	Z	P	Z	P
K	-0.674	0.500	-1.820	0.069
A/Vmax	-0.730	0.465	0.631	0.528
E_{oes}	0.944	0.345	1.540	0.123
$V_{A,eff}$	-1.214	0.225	**-2.521**	**0.012**
$T_{L,CO,'sb'}$	-0.944	0.345	**2.240**	**0.025**
$T_{L/VA}$	0.674	0.500	**2.380**	**0.017**
R_{int}	-0.135	0.893	1.540	0.123
Cst	-0.674	0.500	**-2.100**	**0.036**
pH	1.289	0.197	**2.371**	**0.018**
$PaCO_2$	-0.552	0.581	-1.527	0.127
PaO_2	**-2.032**	**0.042**	-1.260	0.208
HCO_3	**2.032**	**0.042**	**2.521**	**0.012**
tCO_2	**2.023**	**0.043**	**2.521**	**0.012**
BEvt	**2.023**	**0.043**	**2.521**	**0.012**
O_2sat	-1.826	0.068	-1.682	0.092
BP	**-2.023**	**0.043**	**-2.100**	**0.036**
HR	**-2.023**	**0.043**	0.980	0.327
C_{dyn}	**-2.023**	**0.043**	**-2.521**	**0.012**
R_L	1.753	0.080	1.820	0.069

Table 9.2.1 Results of Wilcoxon rank-order tests used to assess whether histamine infusion has a significant effect on cardiorespiratory function. The paired groups used in analysis are the values immediately preceding and immediately following histamine infusion. The statistic Z is the sum of the signed ranks divided by the square root of the sum of the squared ranks. The sign of this statistic indicates the direction of the change. Values in bold type are significant ($P<0.05$).

For seronegative sheep, there were significant reductions in HR, BP, PaO_2 and C_{dyn} and significant increases in HCO_3, tCO_2, and BEvt. With exception to the reduction in HR and PaO_2, these changes also occurred in seropositive sheep. In addition, the seropositive sheep demonstrated a significant increase in $T_{L,CO,'sb'}$, $T_{L/VA}$ and pH and a significant reduction in $V_{A,eff}$ and Cst.

Wilcoxon rank-order tests were repeated on the pooled data i.e. 13 sheep (8 seropositive and 5 seronegative for MVV). The results of this analysis are given in table 9.2.2.

The results of this analysis indicate that for the pooled data there were significant reductions in $V_{A,eff}$, HR, BP, PaO_2, O_2sat, Cst and C_{dyn} and significant increases in $T_{L/VA}$, R_L, pH, HCO_3, tCO_2, and BEvt.

	POOLED DATA (n=13)	
Variable	Z	P
K	-1.782	0.075
A/Vmax	0.118	0.906
E_{oes}	1.503	0.133
$V_{A,eff}$	**-2.900**	**0.004**
$T_{L,CO,'sb'}$	1.363	0.173
$T_{L/VA}$	**2.481**	**0.013**
R_{int}	0.943	0.345
Cst	**-1.992**	**0.046**
pH	**2.805**	**0.005**
$PaCO_2$	-1.339	0.181
PaO_2	**-2.202**	**0.028**
HCO_3	**3.182**	**0.001**
tCO_2	**3.181**	**0.001**
BEvt	**3.180**	**0.001**
O_2sat	**-2.354**	**0.019**
BP	**-2.830**	**0.005**
HR	-0.454	0.650
C_{dyn}	**-3.180**	**0.001**
R_L	**2.481**	**0.013**

Table 9.2.2 Results of Wilcoxon rank-order tests used to assess whether histamine infusion has a significant effect on cardiorespiratory function. The paired groups used in analysis are the values immediately preceding and immediately following histamine infusion. The pooled data set (8 seropositive and 5 seronegative sheep) was used. The statistic Z is the sum of the signed ranks divided by the square root of the sum of the squared ranks. The sign of this statistic indicates the direction of the change. Values in bold type are significant at the levels shown.

The percentage changes induced by the histamine infusion were compared between the two groups by means of the Mann-Whitney U test. The results of this analysis are presented in appendix 9.7. The seropositive group had a significantly greater increase in $T_{L,CO,'sb'}$, $T_{L/VA}$, HCO_3 and BEvt when compared to the seronegative group.

The percentage changes in $V_{A,eff}$, Cst, BP, $T_{L,CO,'sb'}$, and $T_{L/VA}$ in response to histamine infusion are illustrated in figures 9.7.1-9.7.5 respectively.

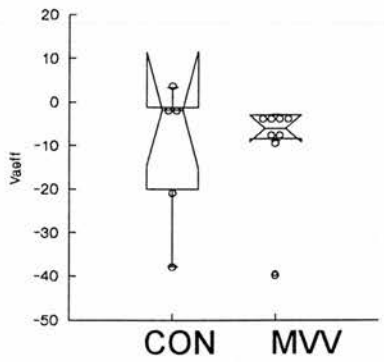


Figure 9.7.1

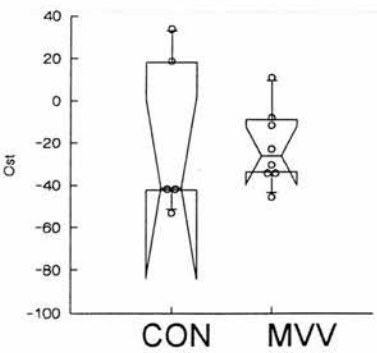


Figure 9.7.2

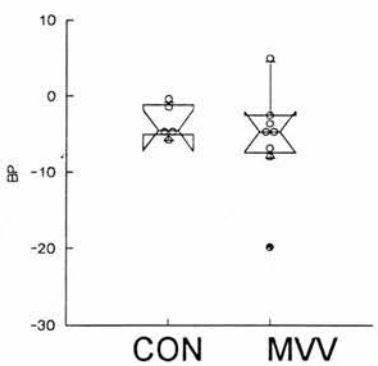


Figure 9.7.3

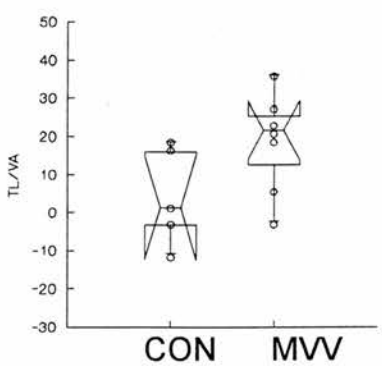


Figure 9.7.4

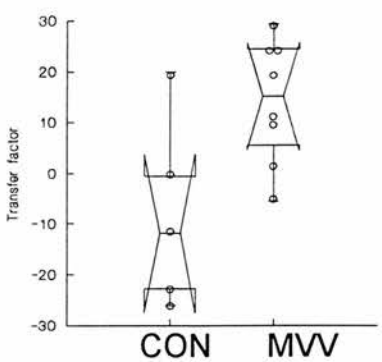


Figure 9.7.5

Summary statistics from morphometric analyses are given in appendix 9.8. Mean V_{vt} values ranged from 18.5 to 29.9% and mean $V_{v'ASMA'}$ values from 1.8 to 3.8%.

The results of Spearman rank-order correlation coefficient tests for testing the association between the functional response to histamine infusion and the quantity of contractile tissue within the lung parenchyma are given in table 9.3. Significant correlations were observed between mean $V_{v'ASMA'}$ and % change in K (r_s = -0.762; P<0.05(two-tailed)), between mean $V_{v'ASMA'}$ and

$ED_{65}C_{dyn}$ (r_s = -0.643; P<0.05(one-tailed)) and between median $V_{v'ASMA'}$ and BP response (r_s = 0.772; P<0.05(two-tailed)). The two former relationships are depicted in figures 9.8 and 9.9.

Variable	V_{vt} mean	V_{vt} median	$V_{v'ASMA'}$ mean	$V_{v'ASMA'}$ median
	r_s	r_s	r_s	r_s
K	-0.429	-0.500	**-0.762**	-0.463
A/Vmax	0.119	0.095	-0.214	-0.129
E_{oes}	0.286	0.286	-0.286	-0.231
$V_{A,eff}$	-0.405	-0.333	-0.333	-0.334
$T_{L/VA}$	0.024	0.071	0.357	0.231
$T_{L,CO,'sb'}$	0.024	0.071	0.357	0.231
R_{int}	-0.143	-0.310	-0.238	-0.154
Cst	0.095	0.095	0.190	-0.129
pH	-0.214	-0.071	0.214	-0.026
$PaCO_2$	0.143	0.000	-0.262	-0.051
PaO_2	0.381	0.262	-0.143	-0.077
HCO_3	0.167	0.119	0.310	0.129
tCO_2	0.238	0.095	-0.024	-0.103
BEvt	0.095	0.119	0.452	0.206
O_2sat	0.476	0.286	0.071	0.180
BP	0.167	0.167	0.714	**0.772**
HR	-0.357	-0.476	-0.143	0.000
R_L	0.071	-0.024	0.333	0.514
$ED_{65}C_{dyn}$	-0.190	-0.190	**-0.643**	-0.617

Table 9.3 Results of Spearman rank-order correlation tests to examine the relationship between the cardiorespiratory response to histamine infusion and lung parenchymal contractile tissue ($V_{v'ASMA'}$). Significant (P<0.05) results are outlined in bold.

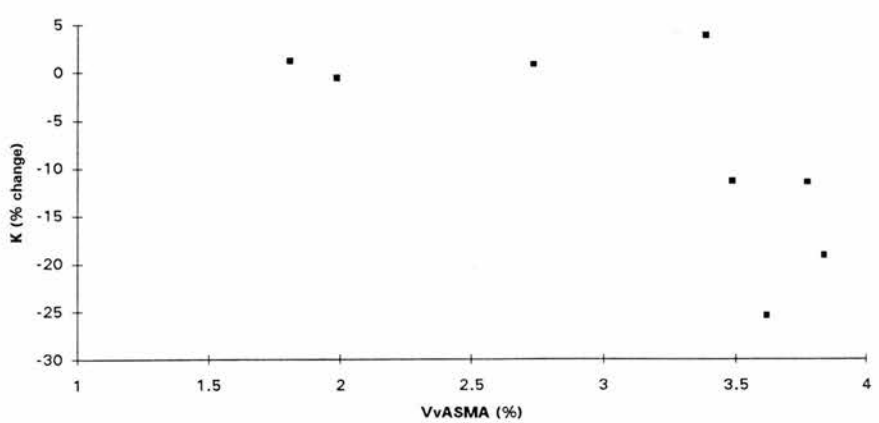


Figure 9.8 Relationship between mean $V_{v'ASMA'}$ and *K* (r_s = -0.762; P<0.05(two-tailed)).

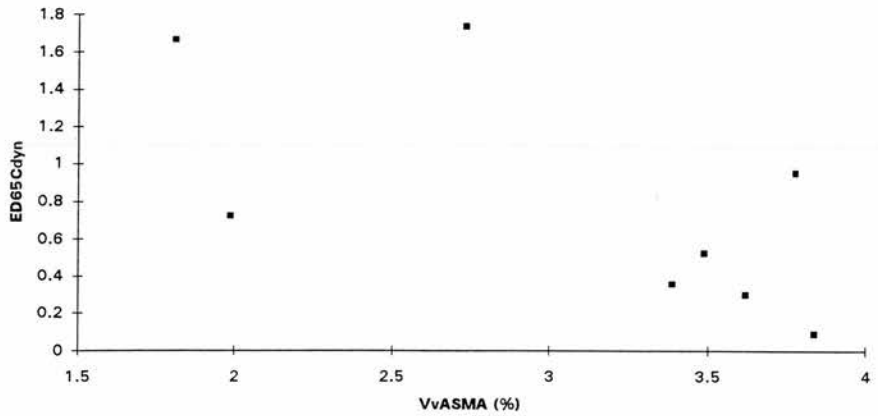


Figure 9.9 Relationship between mean $V_{v'ASMA'}$ and $ED_{65}C_{dyn}$ (r_s = -0.643; P<0.05(one-tailed)).

9.4.2 Clenbuterol injection

The results of both real-time and off-line analysis of data collected just prior to and 15 minutes after an intravenous injection of clenbuterol (0.8μg/kg) are given in appendix 9.9 and appendix 9.10. The results of Wilcoxon signed ranks tests used to assess the significance of changes in these paired data are given in table 9.4.

	Z	P
$V_{A,eff}$	-0.845	0.398
$T_{L,CO,'sb'}$	-0.845	0.398
$T_{L/VA}$	-0.845	0.398
K	**2.201**	**0.028**
Cst	**1.992**	**0.046**
E_{oes}	0.943	0.345
R_{int}	0.845	0.398
pH	1.156	0.248
$PaCO_2$	0.378	0.705
PaO_2	-1.521	0.128
HCO_3	1.693	0.090
tCO_2	**2.197**	**0.028**
BEvt	**2.028**	**0.043**
O_2sat	-1.781	0.075
BP	**-2.201**	**0.028**
R_L	-0.508	0.611
C_{dyn}	-1.690	0.091

Table 9.4 Results of Wilcoxon rank-order tests used to assess whether histamine infusion has a significant effect on cardiorespiratory function. The paired groups used in analysis are the values immediately preceding and immediately following clenbuterol injection. The statistic Z is the sum of the signed ranks divided by the square root of the sum of the squared ranks. The sign of this statistic indicates the direction of the change. Values in bold type are significant (P<0.05).

Significant increases in *K*, Cst, tCO_2 and BEvt and a significant decrease in BP occurred in response to intravenous clenbuterol. Line diagrams of the changes in K and Cst in response to clenbuterol injection are depicted in figures 9.10 & 9.11.

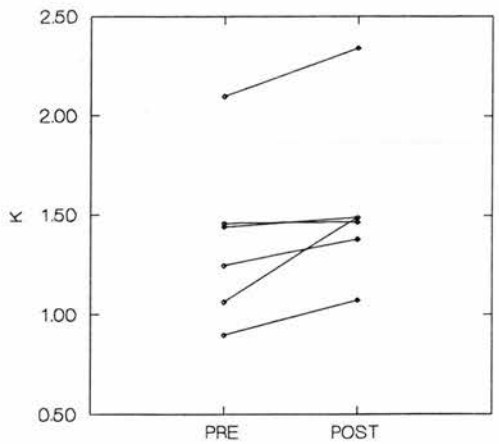


Figure 9.10 Values of K just before and 15 minutes after clenbuterol administration.

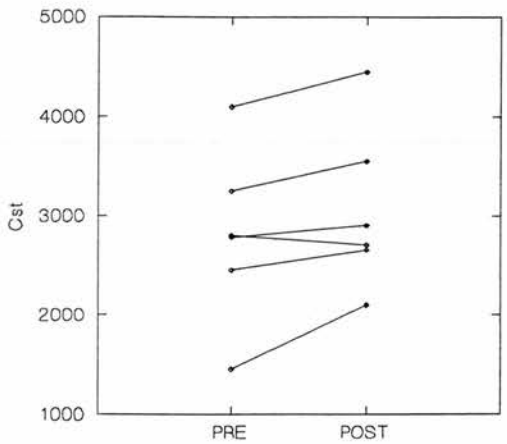


Figure 9.11 Values of Cst (ml/kPa) just before and 15 minutes after clenbuterol administration.

The results of Spearman rank-order correlation tests used to assess the relationship between morphometric variables and the functional response to clenbuterol administration (% change) are given in table 9.5.

Variable		V_{vt} mean	V_{vt} median	$V_{v'ASMA'}$ mean	$V_{v'ASMA'}$ median
	n	r_s	r_s	r_s	r_s
$V_{A,eff}$	7	0.464	0.393	0.250	0.179
$T_{L,CO,'sb'}$	7	-0.429	-0.464	-0.643	-0.677
$T_{L/VA}$	7	-0.714	-0.679	**-0.857**	**-0.797**
K	6	-0.257	-0.029	-0.600	**-0.833**
Cst	6	0.200	0.086	0.314	0.123
E_{oes}	6	-0.771	-0.771	0.676	-0.543
R_{int}	7	-0.321	-0.357	-0.286	0.139
pH	7	0.464	0.393	0.250	0.179
$PaCO_2$	7	-0.519	-0.445	-0.334	-0.124
PaO_2	7	-0.414	-0.270	-0.144	-0.442
HCO_3	7	0.071	-0.071	-0.143	0.020
tCO_2	7	-0.286	-0.429	-0.357	0.060
BEvt	7	-0.393	-0.571	-0.321	0.000
O_2sat	7	-0.414	-0.288	-0.123	-0.392
BP	6	0.371	0.314	0.543	0.414
C_{dyn}	7	0.679	**0.786**	0.536	0.120
R_L	7	**-0.893**	**-0.964**	-0.643	-0.279

Table 9.5 Spearman rank-order correlation coefficients relating to the association between the percentage change in cardiorespiratory functional indices and the quantity of contractile tissue in the lung parenchyma. The one and two-tailed significance levels for n=6 and n=7 are given in table 9.6 below. Significant correlations are indicated in bold type.

	α	0.05	0.025	(one-tailed)
n	α	0.10	0.05	(two-tailed)
6		0.829	0.886	
7		0.714	0.786	

Table 9.6 Critical values of r_s, the Spearman rank-order correlation coefficient.

9.5 DISCUSSION

Choice of histamine as an agonist of peripheral airway constriction was based on the existence of published protocols regarding the administration, and the magnitude and reproducibility of functional effects of this agent in the sheep (Hutchison *et al.*, 1982), together with the existence of published evidence to suggest that the target site of action of intravenously administered histamine is the smooth muscle at the level of the alveolar ducts i.e. the site of interest (Colebatch *et al.*, 1966; Colebatch & Mitchell., 1971). In contrast to histamine, there is a dearth of information regarding the respiratory effects and sites of action of clenbuterol in the lung of sheep. The proposal of this agent as a putative relaxant of peripheral airway contractile tissue was based on published data in humans in which lung elastic recoil was reduced by the intravenous administration of a β-2 agonist (DeTroyer *et al.*, 1978). Another significant influence in the choice of clenbuterol was the knowledge that it was a 'safe' drug to administer to sheep i.e. that, at the levels chosen in this study, untoward toxic effects were unlikely to occur.

Baseline values of mean arterial blood pressure were similar to those previously published for anaesthetized sheep (123 ± 12 mmHg, mean ± S.D.)(Halmagyi & Colebatch, 1961). Baseline values of C_{dyn} and R_L are compared to previously published values in table 9.7. Values of C_{dyn} are in good agreement with previous estimates however values of R_L are considerably greater than all but one previous estimate, that of Coulson *et al.*, (1989). These investigators were able to separate sheep according to baseline lung resistance into high and low resistance groups (table 9.7). The value given for the high resistance group is similar to that found in the present study.

Variable	Unit	n	Mean	S.D.	Anaesthetized?	Source
C_{dyn}	l/kPa	5	0.872	0.533	Y	Present study
C_{dyn}	l/kPa	27	1.081	0.316	Y	Halmagyi & Colebatch, 1961
C_{dyn}	l/kPa	11	0.908	0.275	N	Hutchison *et al.*, 1982
R_L	kPa/l/s	5	0.551	0.030	Y	Present study
R_L	kPa/l/s	11	0.133	0.100	N	Hutchison *et al.*, 1982
R_L	kPa/l/s	6	0.284	0.137	N	Ahmed *et al.*, 1980
R_L	kPa/l/s	19	0.217		Y	Halmagyi & Colebatch, 1961b
R_L	kPa/l/s	5	0.520	0.225	Y	Coulson *et al.*, 1989
R_L	kPa/l/s	6	0.157	0.09	Y	Coulson *et al.*, 1989

Table 9.7 Previously published values regarding C_{dyn} and R_L in sheep. Units have been converted to SI format to facilitate comparison with the present study.

Values of R_{int} were considerably greater than values of R_L. Mead & Whittenberger (1954) identified an 18% difference between the two methods (R_{int}>R_L) and questioned whether chest wall and diaphragm activity damp the change in intrapleural pressure at the point of airway interruption

leading to an overestimation of resistance. Jackson *et al.*, (1974) in a reevaluation of the interrupter technique, argued that if the method of recording Pao is of sufficient resolution, then the changes in Pao due to airway oscillations can be separated from the component due to tissue dynamics (Appendix 9.4) and that this method therefore yields values of airway resistance. The finding in the present study that R_{int} grossly exceeded R_L, despite using high resolution recording equipment and backward extrapolation, does not support the findings of Jackson *et al* (1974). Reservations are therefore expressed regarding interpretation of changes in R_{int} as representative of changes in airway resistance in response to histamine and clenbuterol.

The intravenous administration of histamine and clenbuterol can be expected to influence smooth muscle tone in areas distinct from the lungs. In most instances these extrapulmonary actions will be of no consequence to the study of pulmonary mechanics. However, it is conceivable that these agents could influence oesophageal smooth muscle tone and in this particular instance could invalidate the assumption that oesophageal pressure reflects pleural pressure changes. For this reason, the measurement of oesophageal elastance was included within the experimental protocol. Baseline values of elastance and specific elastance are similar to those reported for conscious unanaesthetized cattle (ranges 0.05-0.20 kPa/ml and 0.49-1.80 kPa/ml/cm respectively (Lekeux *et al.*, 1984)) but considerably lower than those reported for goats (mean values ranging from 0.48 to 1.25 kPa/ml for E_{oes} and from 5.3 to 15 kPa/ml/cm for sE_{oes} (Bakima *et al.*, 1988)). No significant change in oesophageal elastance occurred in response to intravenous infusion of histamine or injection of clenbuterol therefore changes in Ptp were accepted as reflecting changes in lung mechanics.

Histamine infusion appeared to be associated with the development of arterial hypoxaemia (pooled and control data). Arterial hypoxaemia can be caused by inadequate ventilation, by venous admixture through the presence of venous shunt or ventilation-perfusion inequality, or by diffusion impairment (West, 1977). In addition, venous admixture will be strongly influenced by changes in cardiac output (Nunn, 1977). In these sheep the hypoxaemia is unlikely to be caused by hypoventilation as there is no significant change in the $PaCO_2$. In anaesthetized calves given intravenous histamine, Slocombe & Robinson (1981) similarly demonstrated decreased PaO_2 which was caused by an increase in the alveolar-arterial oxygen difference ($\Delta A\text{-}a_{O_2}$). They hypothesized that small airway constriction was causing the increased $\Delta A\text{-}a_{O_2}$ through the development of ventilation-perfusion inequalities. It is suggested that a similar mechanism may account for the observed hypoxaemia in the present study.

In addition to the observed hypoxaemia, the histamine infusion appeared to be associated with the development of an alkalosis. Although the combination of hypoxaemia and eucapnia would tend to argue against a respiratory cause of the alkalosis, examination of the temporal records suggests that the histamine-induced changes in PaO_2 were superimposed upon a gradually developing mild alkalosis that was most likely of respiratory origin i.e. due to hyperventilation. Despite the ventilator rate and tidal volume being adjusted to accepted levels it is entirely possible that this may have occurred. The possibility that mild alterations in acid-base status may have influenced cardiopulmonary function is acknowledged. However, since the direction and magnitude of changes in pH, HCO_3, tCO_2, and BEvt were small and consistent between sheep any influence is assumed to be similar for all sheep.

A reduction in systemic arterial blood pressure in sheep in response to intravenous histamine is consistent with previous studies which have also demonstrated a concurrent increase in pulmonary arterial pressure (Alexander *et al.*, 1967; Kadowitz & Hyman, 1983). Alexander *et al.* (1967) reasoned that the rise in pulmonary arterial pressure was due to constriction of pulmonary vessels and that this, through a reduction in the return of blood to the left side of the heart, was also responsible for the reduced systemic blood pressure. The pulmonary vascular response would presumably lead to pooling of blood within the thorax and pathological and functional evidence to support this was given by Alexander *et al.* (1967). In a study of lung lymph flow in unanaesthetized sheep, Bernard *et al.* (1984), using dose rates of histamine insufficient to cause systemic hypotension, determined that histamine infusion effects a transient (1-3 hr) increase in lung microvascular permeability followed by a sustained increase in microvascular surface area. Although the mechanism of this increase was not elucidated, measurements of single-breath diffusing capacity have frequently been used as an index of the total pulmonary capillary blood volume (Burgess *et al.*, 1968). Indeed, this relationship helps to explain the increase in $T_{L/VA}$ in response to histamine demonstrated in this study (pooled data) i.e. this change reflects the increased microvascular, and hence gas exchanging, surface area in the lung.

One of the most striking differences between the groups was in relation to alterations in gas transfer in response to histamine, namely increased with respect to seropositive sheep and unchanged with respect to seronegative sheep. The low numbers of seronegative sheep included in this study (n=5) precludes accurate interpretation of this difference, however, if an increase in microvascular surface area is accepted for both groups then the reduction in $V_{A,eff}$ noted for the seropositive group will accentuate the differences between the groups in terms of $T_{L/VA}$. Thus an improvement in gas (carbon monoxide) transfer occurs in response to histamine. This might seem paradoxical given the observed hypoxaemia, however the single-breath technique for measuring gas

transfer is insensitive to variance in ventilation-perfusion ratios (Piiper & Sikand, 1966) and would not necessarily show evidence of disturbance in the presence of such inequalities.

Significant reductions in $V_{A,eff}$ and Cst and a trend towards a reduction in K were apparent for the pooled data and for the seropositive sheep. These results suggest that static lung volumes and mechanics are influenced by histamine infusion. What is the mechanism of this change? Lung volume at a given Ptp will be influenced by lung and chest wall elastic properties and by the volume of thoracic structures. With respect to the latter, an obvious possibility is that pooling of blood in the pulmonary vascular system is responsible for reducing lung volume through a tissue filling effect, with additional direct and indirect effects on lung elastic properties. In studies of the effect of pulmonary vascular pressures on single-breath transfer factor in anaesthetized dogs (Burgess *et al.*, 1968; Karp *et al.*, 1968) large changes in transfer factor in response to alterations in pulmonary arterial or left atrial pressure were demonstrated. However, no changes in alveolar volume measured using an inert gas dilution technique similar to that described in the present study were demonstrated in the former study (Burgess *et al.*, 1968). Further, during water immersion there is a redistribution of blood volume to cause a central hypervolaemia (Begin *et al.*, 1976) and this leads to an increase in pulmonary capillary blood volume and $T_{L/VA}$. Despite this, no significant increase in pulmonary tissue plus capillary volume could be demonstrated using a rebreathing method in immersed human subjects (Begin *et al.*, 1976). Thus, although logic suggests that the histamine-induced increase in pulmonary capillary surface will contribute to a reciprocal reduction in alveolar volume, the relative magnitudes of change in alveolar volume when compared to the likely volume change of the pulmonary capillary blood volume (Appendix 9.11) suggest that other factors must make a significant contribution.

Pulmonary vascular congestion may also influence lung compliance. Borst *et al.*, (1957) studied the effect of raising pulmonary arterial and left atrial blood pressures on lung compliance in anaesthetized dogs. The latter, but not the former, was associated with a reduction in lung compliance proportional to the increase in pressure. The effect i.e. pulmonary venous engorgement, is similar to that produced in humans by water immersion or inflation of an anti-gravity suit (Bondurant *et al.*, 1957). Alterations in pulmonary haemodynamics were considerable in these studies and contrasts to the probably mild increase in pulmonary vascular volume brought about by histamine infusion in sheep in this study (Appendix 9.11). Thus, it is concluded that the increase in pulmonary capillary blood volume will contribute towards a 'stiffening' of lung parenchyma and a reduction in static and dynamic compliance. However, the extent of change in lung elastic properties is unlikely to be wholly accounted for by the alterations in pulmonary vascular status in response to histamine infusion.

It is hypothesized that, in this study, the principal cause of the significant histamine-induced reductions in $V_{A,eff}$ and Cst and trend towards a reduction in K (seropositive and pooled data) is the contraction of parenchymal contractile tissue at the level of the alveolar ducts. Evidence from previous studies supports this hypothesis. In a study of the effects of barium sulphate embolism in anaesthetized cats, Nadel *et al.*, (1964) demonstrated a reduction in lung compliance, and an increase in resistance and dead space. These changes were associated with a 'pulling-in' of the alveolar septal tips towards the centre of the ducts with the degree of constriction being correlated with the level of change in pulmonary mechanics. An increased stiffness of the alveolar ducts secondary to increased muscle tone could be responsible for the reduction in lung compliance. That histamine was a likely mediator of this reaction was demonstrated in a subsequent study of the effects of histamine infusion into the right side of the heart of anaesthetized cats (Colebatch *et al.*, 1966). Again anatomic studies demonstrated a 'pulling-in' of the alveolar septal tips towards the centre of the alveolar ducts. Functional consequences were an increase in the expired volume associated with a reduced compliance. Changes in compliance usually preceded, and indeed often occurred in the absence of, changes in lung resistance. Colebatch & Mitchell (1971), in a study of the effects of histamine on liquid-filled cat lungs, reasoned that the magnitude of increase in elastic recoil and reduction in lung volume could not be explained by airway narrowing or closure or by surface forces. Changes in lung volume of 27% and greater in cat lungs could only be explained if the size of alveoli was reduced i.e. constriction of the alveolar ducts alone could not account for this reduction. These authors suggested that alveolar volume would be reduced by duct constriction if the duct smooth muscle acts in series with the passive fibre network within the lungs. Indeed histamine was capable of virtually emptying the respiratory units in the liquid-filled preparations at zero pressure (Colebatch & Mitchell, 1971). In this latter study, the relative abundance of smooth muscle in the alveolar ducts was visually assessed and appeared to correlate with the degree of volume reduction.

If similar mechanisms operate within sheep lungs in response to histamine then a correlation would be expected between the response to histamine infusion and the quantity of parenchymal contractile tissue. The results of this study indicate that such correlations exist. The dose required to lower C_{dyn} to 65% of baseline values was negatively correlated with the quantity of ASMA, indicating that sheep with increased parenchymal contractile tissue show proportionally greater responses to equivalent doses of histamine. Similarly, the percentage change in K was correlated with the quantity of ASMA indicating that the more contractile tissue within the parenchyma, the greater the effect a given dose of histamine would have on lung distensibility. Argument could be presented that the changes that are elicited by histamine are mediated through airway constriction and/or closure, in that Mitzner *et al.* (1992) showed that changes in lung compliance in anaesthetized sheep in response to methacholine could be caused by central airway

constriction with no peripheral airway constriction. With such changes in central airway calibre, changes in R_L and R_{int} would be anticipated. Although, as previously intimated, the interpretation of R_{int} is subject to considerable qualification, in this study there was only one instance in which there was a twofold increase in R_L in response to histamine and for the separate groups no significant changes in either parameter occurred in response to histamine. Additionally, argument could be presented that the reduction of lung volume in reponse to histamine is a feature of airway closure and gas trapping. Given that lung volume was measured at a Ptp of 3kPa, it is suggested that all airways would be open at this Ptp and all gas exchange units would be in communication with the inert gas mixture thus giving an accurate assessment of $V_{A,eff}$. That this is a valid assumption has recently been questioned (Merkus & Quanjer, 1992) however given the extent of reduction in C_{dyn} with no concomitant change in R_L, it is considered unlikely that this phenomenom accounts for significant volume reduction in this study.

The correlation between percentage reduction in systemic blood pressure and $V_{v'ASMA'}$ is more difficult to explain. If the hypothesis relating the fall in systemic blood pressure to pulmonary vessel constriction is correct then a link is suggested. Although subjective opinion is that there is negligible increase in vascular smooth muscle in maedi, in the morphometric analysis no attempt was made to quantify the different types of contractile tissue within the parenchyma. It is therefore possible that histamine, in addition to the effects on alveolar ductal smooth muscle, was also constricting pulmonary vascular elements and that the consequences of this constriction were relatively greater for sheep with more vascular smooth muscle.

The magnitude of reduction of K in maedi can be considerable (chapter 4). However, the extent of reduction in K in response to histamine for seropositive sheep (+3.9 to -25%) indicates that contraction of parenchymal smooth muscle is at least capable of causing changes in K of similar magnitude. In chapter 8, although an association between $V_{v'ASMA'}$ and K was demonstrated, the strong interdependence between $V_{v'ASMA'}$ and V_{vt} prevented a definitive interpretation of this link. In these studies, no correlation was apparent between any of the functional responses and V_{vt} thus crucially identifying $V_{v'ASMA'}$ as a dynamic component within the parenchyma individually and strongly associated with a reduction in K. In the light of these studies of the cardiopulmonary response to histamine infusion it is suggested that the increase in ASMA demonstrated in maedi, is of functional relevance and is responsible for the reduction in lung distensibility.

With regard to the effects of clenbuterol injection, 15 minutes was selected as an appropriate interval to allow for any measurable influence on cardiopulmonary function. This time interval was based on studies in anesthetized horses where clenbuterol results in changes in

cardiopulmonary function within 5 minutes of administration and these changes persist for 90 minutes (Keegan *et al.*, 1991).

Intravenous clenbuterol injection was associated with significant increases in *K*, Cst, tCO_2 and BEvt and a significant decrease in BP. Changes in acid-base parameters are probably a reflection of the development of a mild respiratory alkalosis as identified previously for sheep undergoing histamine infusions. The reduction in systemic blood pressure was unexpected, given that β-agonists are often used for their positive inotropic effects (Weiner, 1985). However, orally administered clenbuterol caused an increase in heart rate and a similar reduction in mean arterial pressure in sheep (Brockway *et al.*, 1987). Thus clenbuterol appears to exert both β-1 and β-2 agonistic effects in that it causes both cardiac stimulation (β-1) and vasodepression (β-2)(Brockway *et al.*, 1987). The changes in static mechanical properties were unaccompanied by significant changes in blood gases, $V_{A,eff}$ or R_L. DeTroyer *et al.*, (1978) demonstrated an increase in conductance and a reduction in lung elastic recoil in response to intravenous infusion of β-agonist in humans. These authors attributed the changes in lung recoil to the dilation of alveolar duct smooth muscle, excluding large airway influence largely on the basis of temporal differences in action. In a study of the effect of intravenous atropine on the static mechanical properties of human lungs, Crawford *et al.*, (1987) demonstrated a decrease in lung elastic recoil but no change in *K*. These changes, caused by a reduction in bronchomotor tone, were associated with increased ventilation maldistribution.

Of the variables that were significantly altered by clenbuterol infusion, only *K* showed a correlation with $V_{v'ASMA'}$. This correlation was such that sheep showing the greatest increase in K had the lowest $V_{v'ASMA'}$ values. If parenchymal contractile tissue determines lung distensibility and clenbuterol causes relaxation of this tissue element then it would be reasonable to assume that the dose-effect relationship would have been related to the quantity of contractile tissue. The observed relationship is the opposite to that anticipated i.e. the more contractile tissue, the less the increase in *K*. Two possible explanations could account for these findings. The first would be that the clenbuterol is not acting on the alveolar duct smooth muscle, but rather on other contractile tissue, such as that surrounding the conducting airways. Such a site of action could still be capable of causing changes in lung elastic properties (Crawford *et al.*, 1987) and would in addition contribute to considerable inhomogeneity in ventilation distribution. If this is the case then the dynamic elastic properties of the lungs will be determined by a balance between airway and parenchymal contractile tissues. This is an interesting hypothesis as it would to some extent account for the relationship shown i.e. if the parenchymal tissue fails to relax in response to clenbuterol then the change in K will be less for the sheep with the greatest amount of contractile tissue, this being the observed

relationship. Against this hypothesis is the failure to demonstrate any significant reduction in R_L or R_{int} (recognising the limitations of the latter measurement). The second, less appealing, hypothesis would be that the alveolar duct muscle is already in a relaxed state and therefore incapable of further reduction in tone. Against this hypothesis are the observed changes in Cst and *K* in response to clenbuterol and the lack of an adequate explanation to account for the correlation between K and $V_{v'ASMA'}$. Other significant correlations existed between percentage change in functional variables and morphometric indices ($T_{L/VA}$.vs $V_{v'ASMA'}$ and C_{dyn} and R_L vs V_{vt}). However, the fact that no significant changes in these variables could be demonstrated for the group and the small overall group size precludes detailed analysis of these changes.

9.6 SUMMARY

It was hypothesized that the quantity of contractile tissue at the level of the alveolar ducts in lung parenchyma is a principal determinant of the lung elastic properties in maedi and is responsible for the reduction in lung distensibility seen in this condition. In order to separate the influence of dynamic and passive tissue elements, pharmacological mediators were administered to anaesthetized sheep, both seropositive and seronegative for MVV, in an attempt to cause relaxation and contraction of parenchymal contractile tissue i.e. the dynamic element. The functional response of the cardiopulmonary system to intravenous infusion of histamine and intravenous injection of clenbuterol was therefore measured and correlated with morphometric estimates of the quantity of contractile tissue within the pulmonary parenchyma. The dose of histamine required to lower dynamic compliance to 65% of baseline values was negatively correlated with the quantity of contractile tissue within the lung parenchyma indicating that sheep with increased parenchymal contractile tissue show proportionally greater responses to equivalent doses of histamine. Similarly, the percentage change in lung distensibility was correlated with the quantity of parenchymal contractile tissue, indicating that the more contractile tissue within the parenchyma, the greater the effect a given dose of histamine would have on lung distensibility. These findings support the contention that parenchymal contractile tissue is of functional relevance and capable of regulating overall lung elastic properties. Lung distensibility and static lung compliance was significantly increased following clenbuterol injection. Of these changes, only the increase in distensibility was correlated with the quantity of parenchymal contractile tissue. The correlation was negative, indicating that sheep with large increases in distensibility in response to clenbuterol had less parenchymal contractile tissue. These results could be explained if the site of action of clenbuterol was not the contractile tissue at the level of the alveolar ducts, but rather that which surrounds conducting airways. However, it should be understood that this latter explanation simply represents a reasoned hypothesis based on a subjective interpretation of the present results.

CHAPTER 10

FINAL DISCUSSION

The purpose of this closing chapter is to broadly discuss and evaluate the results of this thesis both in relation to initial objectives and with specific regard to areas where future relevant research is indicated.

The overall objectives of this thesis were (a) to specifically define maedi in functional terms, (b) to identify structural correlates of functional abnormalities, and (c) to elucidate the pathophysiological mechanisms that are responsible for observed correlations. A principal reason for defining these objectives was the need to develop techniques that would allow the objective quantitation of maedi in the preclinical phase such that *in vitro* investigations at the cellular level could be related to the *in vivo* sequence of events. To what extent has this study satisfied these needs?

The foundation of this thesis was the development and application of techniques necessary for the study of lung function in sheep. Thereafter an investigation of maedi was undertaken in which the disease was functionally defined. Following characterisation of the functional abnormality and correlation with pathological findings, relationships were identified that could not be explained under existing dogma. An experimental hypothesis was proposed to account for the observed findings, and this hypothesis was tested in the last two chapters. Discussion and evaluation of this thesis will follow the lines of this investigation.

The relative lack of information regarding lung function parameters in anaesthetized sheep (Chapter 1), the possible contribution of breed-related influences and the inter-laboratory variation in quoted values for lung function indices (Chapter 9; section 9.5.2) prompted a study of lung function in normal sheep of similar breed, age and sex to the sheep from the parent flock available for investigation (Chapter 1). This study was particularly comprehensive in terms of numbers of sheep studied, the number of repeat determinations made and the period over which the study was conducted. As such, the data represents a valuable reference point for sheep pulmonary research in health and disease and for comparative respiratory research in general. In the context of this thesis, this fundamental step allowed the setting of normal limits for relevant lung functional tests based on bodyweight measurements (Chapter 1), and the value of this preliminary investigation was manifest throughout the thesis in that predicted values and confidence limits could be established for any individual or group of sheep within the infected flock. Moreover, time sequential changes in lung

function in infected sheep could be assessed and the significance of such changes interpreted with confidence. Likewise, the detailed analysis and characterisation of the sheep lung pressure-volume curve (Chapter 3) represents the first comprehensive study of this nature in the literature. Whilst not representing a novel approach, this study provided the means to assess lung elastic properties in maedi in the knowledge that measured changes were a true reflection of the same and not simply a reflection of the volume reduction characteristic of this condition. This analysis was of particular value in identifying the apparently paradoxical relationship between surface density and lung distensibility in chapter 7.

The sensitivity of lung compliance, lung volume and transfer factor measurements in detecting pathological change in maedi was assessed through a correlative study of lung structure and function (Chapter 7). This correlative approach has been frequently employed in humans and animals where functional test measurements have been compared to subjective or objective estimates of pathology, as assessed by lung biopsy or necropsy procedures (Gaensler *et al.*, 1975; Carrington *et al.*, 1976a,b; Huang *et al.*, 1979; Fulmer *et al.*, 1979; Berend *et al.*, 1985). The present study is unique in two respects, firstly, to the author's knowledge, this is the first pathophysiological study in the sheep to use quantitative morphometry of the whole lung as the means of assessing the progression of interstitial lung disease, and secondly, this is the first attempt to investigate structure-function correlations in a naturally occurring lung disease of sheep.

Indices of gas exchange were the most sensitive of the functional measurements in detecting pathological changes in maedi (Chapter 7). Indeed, indications were that reduced gas exchanging capabilities actually predated structural pathology. However, this correlative approach simply results in the tests being relative-ranked according to sensitivity and offers no information regarding the within-test accuracy in quantifying pathology. It is this latter question which is of crucial importance if lung function analysis has a role to play in integrated research into maedi pathogenesis i.e. the predictive capability of lung function analysis in determining the extent of pathology is a central issue.

Statistically, regression analysis is used to predict one variable from another. In using regression analysis certain assumptions regarding the distribution of the dependant variables (morphometric indices) are made, namely that the distribution of these variables at each value of the independent variable (functional measurements) are normal with the same variance. No assumptions are made about the independent variable (Gardner & Altman, 1989). It was considered unlikely that the size and spread of the morphometric values in the data set examined would allow the above distributional assumptions to be met and secondly would allow the generation of useful prediction

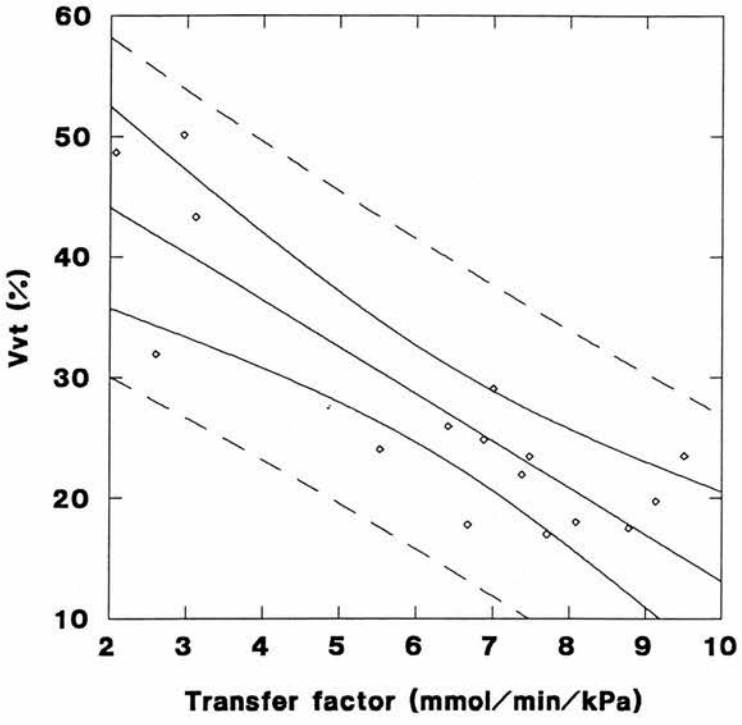


Figure 10.1 Graph showing relationship between transfer factor for carbon monoxide ($T_{L,CO,'sb'}$) and tissue volume fraction (V_{vt})(r_s= -0.721;P<0.001). The solid straight line is the line of least squares linear regression, the inner solid curved lines represent the 95% confidence interval for the mean value of V_{vt} for a given sample mean value of $T_{L,CO,'sb'}$ and the dashed lines represent the 95% prediction intervals for an individual V_{vt} value based on a given value of $T_{L,CO,'sb'}$

confidence inervals. However, an indication of the potential predictive value of lung function tests can be realised by completing linear regression analysis on the limited data set used in the pathophysiological correlative study (Chapter 7) and further calculating confidence intervals where distributional assumptions regarding the error terms appear to be met. This illustration is only likely to be relevant where correlations are strong and for the purposes of this discussion is therefore restricted to the relationship between transfer factor ($T_{L,CO,'sb'}$) and tissue volume fraction (V_{vt})(r_s= -0.721;P<0.001). The plot of this relationship together with fitted linear regression line and confidence intervals is given in figure 10.1 (facing). The straight solid line is the line of least squares linear regression, the inner solid curved lines represent the 95% confidence interval for the mean value of V_{vt} for a given sample mean value of $T_{L,CO,'sb'}$ and the dashed lines represent the 95% prediction intervals for an individual V_{vt} value based on a given value of $T_{L,CO,'sb'}$. As can be appreciated the prediction intervals are very wide and reflect the considerable scatter of data around the regression line. Another factor that might contribute to the relatively wide prediction intervals relates to the nature of the association that is being demonstrated i.e. if dysfunction in gas exchange predates structural pathology (as is suggested in chapter 7) and is, at least in the early stages of maedi, related to changes in gas exchange units at the ultrastructural or molecular level, then it would be unrealistic to expect narrower prediction intervals than the above to be demonstrated. As such the predictive value of individual transfer factor measurements must remain rather limited until the data set expands and the spread of residuals is reduced. Foreseeable use of the present data can however extend to the prediction of population mean values for V_{vt} based on sample mean values of $T_{L,CO,'sb'}$. This might allow the grouping of animals according to functional impairment and with a degree of confidence that the group is derived from a population with given morphometric characteristics - an obvious advantage where there is a desire to compare, pre-mortem, biological samples derived from groups with different stages of lung pathology.

Although a detailed analysis was made of the sensitivity of lung function analysis, no attempt was made to assess the specificity of the same in detecting maedi. Two primary reasons contributed to exclude such an assessment from this study. The first relates to findings in human respiratory medicine, where lung function indices such as lung compliance, lung volume and transfer factor measurements are recognised as being reasonably sensitive but of poor specificity in detecting different interstitial lung diseases (Gaensler *et al.*, 1975). It was therefore considered unlikely that a different situation would hold for the sheep and that the tests of functional disturbance used in this study would be unlikely to accurately differentiate between maedi and other interstitial lung diseases e.g. sheep pulmonary adenomatosis, or be able to detect complicated mixed pathologies e.g. maedi complicated by the presence of localized parenchymal abscessation. The second reason relates to the fortunate lack of complicating pathologies that occur within the parent flock used for these studies (Watt *et al.*, 1992), thereby minimizing the need to accurately diagnose

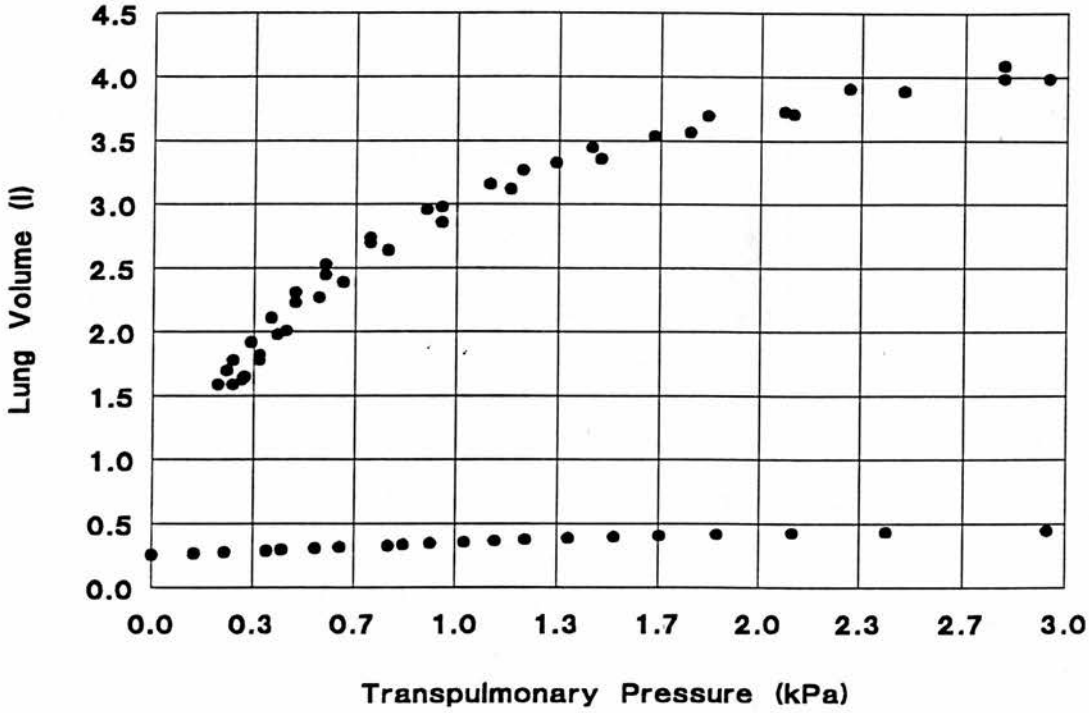


Figure 10.2 Figure showing sheep lung pressure-volume curves generated *in vivo* and *ex vivo*. the excised lung pressure-volume curve was obtained immediately after euthanasia. The lung was inflated in uniform incremental steps using a syringe connected to the tracheal opening. The airway opening pressure was monitored with a differential pressure transducer (CS9; Mercury Electronics). The volume of gas in the collapsed lung was estimated by accurately weighing the collapsed lung, then blocking the tracheal opening and measuring lung volume by water displacement. The specific gravity of the lung tissue was assumed to be 1 gm/cm^3.

the presence and extent of other pathologies within the flock pre-mortem. Indeed, it would have been virtually impossible to assemble a data set to include representative numbers of the more commonly detected lung pathologies of sheep and yet still satisfy the preconditions regarding breed, age and sex that applied to the sheep used in the present study. Given the assumed lack of specificity of the tests of functional disturbance used in this study it should be recognised that, where necropsy is unavailable to confirm or refute diagnosis, these tests should be interpreted in conjunction with whatever other ancillary diagnostic aids are available, such as clinical findings, radiography, bronchoalveolar lavage and routine haematology.

The demonstration that the time between euthanasia and inflation fixation of the lungs is a determinant of the ratio of fixed to physiological lung volume (Chapter 6) has implications for the interpretation of quantitative morphometric data both within and between studies. That there is indeed a considerable change in the elastic properties of the lungs *ex vivo* is indicated in figure 10.2 (facing page). The two curves represent pressure-volume curves of ovine lungs obtained both *in vivo* and *ex vivo*. As can be appreciated there is a phenomenal restriction on the degree of lung expansion *ex vivo*.The mechanism behind this relationship deserves further study. A structured investigation of time sequential changes in the pressure-volume curves of excised ovine lungs as described by Lai *et al.* (1984) for guinea pig lungs is warranted for the ovine lung.

Transfer factor measurements appeared to be the most reproducible and sensitive in detecting abnormal lung function following MVV-infection (Chapters 2 & 7). The possibility that a similar relationship exists between MVV-specific cytotoxic T-lymphocytosis and lung epithelial permeability as has been demonstrated for HIV-infection (Meignan *et al.*, 1990) is attractive and would explain the apparent decrease in transfer factor prior to significant structural abnormalities being detected (Chapter 7). A further observation by Meignan *et al.* (1990) was that patients with advanced HIV-disease frequently had a lymphocytic alveolitis without changes in epithelial permeability. This alveolitis was characterized by CD8 natural killer cells, not cytotoxic T-lymphocytes. Meignan *et al.*, (1990) suggested that release of mediators by alveolar macrophages targeted by cytotoxic T-lymphocytes such as interleukin-1 (IL-1), intracellular enzymes, free radicals or tumour necrosis factor-α (TNFα) might contribute towards damage to alveolar epithelial damage. Alternatively a direct effect throught the release of proteolytic substances or cytokines by cytotoxic T-lymphocytes might play a role in this regard (Meignan *et al.*, 1990). Certainly, TNFα is an attractive candidate for causing the type of local damage implicated. The major cellular source of TNFα is the activated mononuclear phagocyte (Abbas *et al.*, 1991) and the effects of this cytokine on the lung parenchyma are thought to be largely dependant on level, with low levels perpetuating chronic inflammatory processes (Wewers & Gadek, 1991). At the cellular level TNFα activates

neutrophils and eosinophils, causing increased phagocytosis, endothelial adherence, degranulation, and elaboration of toxic oxygen species (Abbas *et al.*, 1991; Wheeler *et al.*, 1990). TNFα also causes the production of IL-1 by macrophages and endothelial cells and interleukin-2 (IL-2) by activated lymphocytes thus providing the mechanism for further amplification of the immune response (Abbas *et al.*, 1991; Wheeler *et al.*, 1990). Many of the physiological effects of TNFα are thought to be mediated through cyclooxygenase products and administration of inhibitors of these products (e.g. ibuprofen) leads to improved physiology (Wheeler *et al.*, 1992).

These observations encourage further elucidation of the pathophysiological mechanisms contributing to the reduction in gas exchanging ability in maedi. Further correlative studies linking functional changes to the CD8-lymphocytosis that is a feature of maedi (Cordier *et al.*, 1992; Luján *et al.*, 1993) are required to establish this link. The result of assays to quantify the cytotoxic activity of BAL lymphocytes against autologous alveolar macrophages could be correlated with functional deficits in gas exchange. Further, these studies could be extended to determine whether temporal phenotypic modulation of the BAL lymphocyte population during progression of maedi correlates with functional deficits in gas exchange. The role of the neutrophil in mediating lung epithelial damage and promoting the inflammatory response is also a relevant area for future research given the observed neutrophilia in BALF from MVV-infected sheep (Cordier *et al.*, 1992; Luján *et al.*, 1993). The levels of cytokines such as TNF that are proposed to play a role in damaging lung epithelium could be assessed by biological assay or quantitative ELISA in BALF, although such examination might prove insensitive to quantifying what is probably very localized secretions of these mediators. Immunocytochemical and molecular studies such as polymerase chain reaction or in situ hybridization, would be better placed to provide relevant information regarding quantitative and qualitative aspects of cytokine expression at the local cellular level. It is tempting to speculate that, should TNF be identified as playing a role in causing chronic damage to the lung epithelium, then administration of cyclooxygenase products might have a beneficial effect on gas exchange.

The results of chapter 7 indicated that tissue forces might play a role in limiting lung distensibility in maedi. It was hypothesized that the increase in parenchymal contractile tissue that occurs in maedi (chapter 8) might play a role in this regard and by its nature, this hypothesis could only be tested *in vivo*.

The approach used in chapter 9 to determine the functional significance of parenchymal contractile tissue was to focus on the functional effects of contraction and relaxation of this element. The results of this and the preceding study (Chapter 8) indicated that lung distensibility is related to the quantity and functional tone of parenchymal contractile tissue, thereby strongly implicating this

element as the source of the tissue forces responsible for the reduction in lung distensibility seen in maedi. These studies represent the first conclusive evidence that, in the sheep, the response to intravenous histamine is related to the quantity of parenchymal smooth muscle and this finding has implications regarding the use and interpretation of tests of airway hyperresponsiveness in this species. Argument could be advanced that, in the case of the histamine infusion, no direct evidence was presented that this agonist caused contraction of parenchymal ducts and small peripheral airways. This argument is valid and although circumstantial functional measurements suggested the contraction of this element, the only direct way of demonstrating peripheral 'pneumoconstriction' would be by open lung biopsy during infusion with immediate immersion of the biopsy in liquid nirogen and subsequent processing and examination by light or electron microscope (Mitzner *et al.*, 1992). Similar arguments could be presented regarding the effects of clenbuterol, although in this instance, the functional effects were not suggestive of relaxation of the parenchymal contractile tissue. Again, direct evidence to support this hypothesis could only be derived by open lung biopsy following administration of clenbuterol.

Parenchymal tissue related forces other than those associated with dynamic contractile tissue elements relate to the noncellular elements i.e. the passive interstitial tissue network of collagen and elastic fibres. The role of this passive network was not investigated in this thesis. Georgsson *et al.* (1976) described elastic fibres to be sparse and fragmented in maedi. The presumed effect of this disturbance to the passive fibre network would be a reduction in lung elastic recoil, not the increase that is in fact observed (chapters 4 & 7). It is interesting to note that in SIV-infected macaques, an arteriopathy is described in which there is significant fragmentation and disruption of the elastic fibres in the elastic laminae (Chalifoux *et al.*, 1992). Speculating endothelial damage as the progenitor step, these authors hypothesized that the damage to the elastic fibres was the result of inflammatory cytokine and/or protease release. Although recognised to occur, arteriopathy in lungs is not a significant feature in maedi (Georgsson & Palsson, 1971). However, microscopic arterial lesions have been found in joint capsules, kidneys, meninges and brains of sheep with ovine progressive pneumonia, a virtually identical disease to maedi-visna caused by a lentivirus with indistinguishable physical properties to those of MVV (Cutlip et al., 1979,1985). The coincidental nature of the pathology affecting the elastic fibres in human and ovine lentiviral diseases is of interest. It is unclear whether the disordered elastic fibre morphology is solely a consequence of destructive processes or whether there is also a component of disorder arising as a result of abnormal synthetic pathways. Initial direct damage to fibres could be caused by the release of elastases by neutrophils or lymphocytes, both cell types being increased in BALF in maedi (Cordier *et al.*, 1992; Luján *et al.*, 1993). Under normal circumstances there is very little remodelling of elastic fibres during adult life, however under such conditions of fibre destruction, reactivation of synthetic pathways can be anticipated. Although the myofibroblast is considered the source of elastin during

lung development, it is not known which cell type takes over this responsibility in the adult lung. Given that the elastic fibres of the axial fibre skeleton at the level of the alveolar duct are distributed in an almost identical pattern to that of contractile cells in normal lung (Weibel & Bachofen, 1991; Chapter 8), it is tempting to speculate that these cells or cells in the immediate locality are involved. The nature of the involvement is entirely hypothetical, however, given that mechanical stress might play a role in inducing ASMA expression by lung parenchymal cells (Kapanci *et al.*, 1990), the observed increase in ASMA expression in maedi might be related to altered micromechanical stresses within the extracellular matrix (ECM) caused by the fragmentation and disruption of the elastic fibres. Such a mechanism could conceivably be mediated through transmembrane receptors and signal transduction pathways, or through direct transmission of ECM forces to intracellular cytoskeletal elements (Bissel *et al.*, 1982; Ingber & Jamieson, 1985) so as to alter the pattern of gene expression in local cells. Alternatively, a more indirect pathway involving the local expression and release of cytokines may induce the ASMA expression. These intriguing possibilities warrant further investigation, however, a priority must remain the morphometric assessment of the elastic fibre skeleton in maedi lungs. Although there are difficulties in fixing and staining elastic tissue under well-controlled functional conditions (Oldmixon *et al.*, 1985) an initial histological assessment using morphometric techniques such as those demonstrated by Niewoehner & Kleinerman (1977) would probably indicate the extent of fibre disruption. A related functional approach would be to determine, through the study of pressure-volume curves at a suitable time point after euthanasia, whether there is any loss of elastic recoil in excised maedi lungs.

These and other questions that have arisen through this study of pathophysiological correlations in maedi indicate the value of *in vivo* quantitative lung function assessment both as a powerful investigative tool in its own right and as a potentially valuable means of integrating *in vitro* molecular and cellular biological studies with the source of all events, the animal itself.

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APPENDIX

Appendix 2.1 Description of sheep used to formulate normal Cst, $V_{A,eff}$, $T_{L,CO,'sb'}$ and $T_{L/VA}$ values and to determine the reproducibility of these measurements. Indicated bodyweights were those determined just prior to commencement of the study.

Sheep No.	Bodyweight (kg)	Breed	Sex	Year of Birth	Serological Status	
					Result	Test Date
CON038	59	TEXEL	F	1983	-ve	25/1/91
CON040	86	TEXEL	F	1986	-ve	25/1/91
CON042	70	TEXEL	F	1986	-ve	25/1/91
CON043	58	TEXEL	F	1986	-ve	25/1/91
CON088	63	TEXEL	F	1986	-ve	25/1/91
CON089	71	TEXEL	F	1986	-ve	25/1/91
CON090	65	TEXEL	F	1984	-ve	25/1/91
CON091	82	TEXEL	F	1984	-ve	25/1/91
CON092	85	TEXEL	F	1985	-ve	25/1/91
CON093	63	TEXEL	F	1984	-ve	25/1/91
CON094	52	TEXEL	F	1989	-ve	25/1/91
CON095	62	TEXEL	F	1984	-ve	25/1/91
CON096	79	TEXEL	F	1985	-ve	25/1/91
CON097	57	TEXEL	F	1984	-ve	25/1/91
CON098	87	TEXEL	F	1984	-ve	25/1/91
CON099	53	TEXEL	F	1985	-ve	25/1/91

Appendix 2.2 Anatomic and instrumental deadspace

Anatomic deadspace

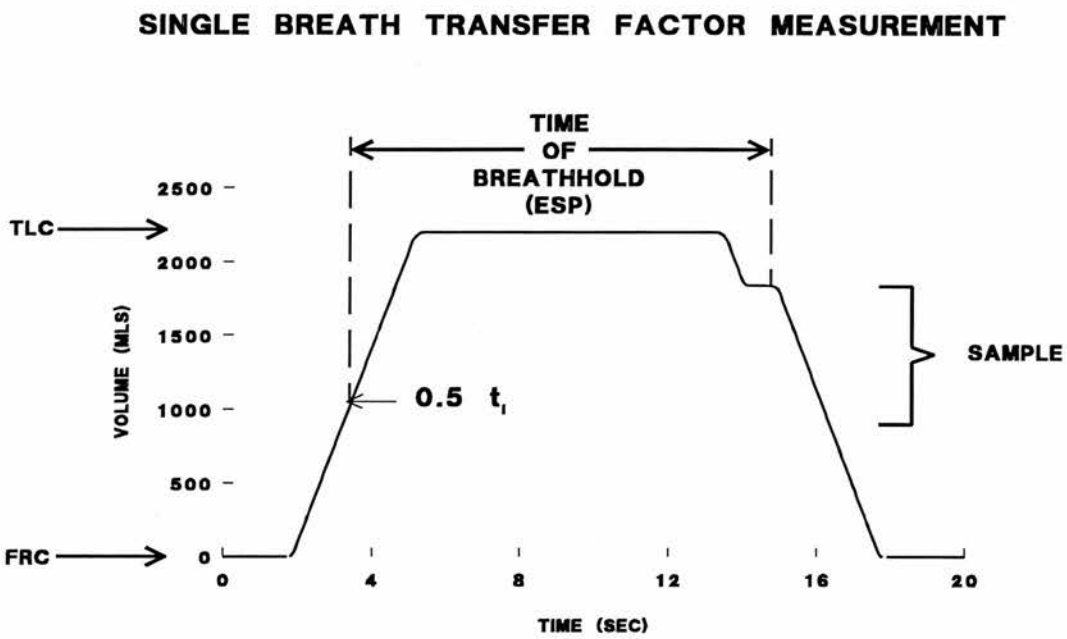
Relevant anatomic deadspace refers to the conducting airways between the balloon cuff of the endotracheal tube and the respiratory bronchioles. It is assumed that Stahl's (1967) power law formulae for anatomic deadspace refers to both upper and lower respiratory tract deadspace and therefore would overestimate anatomic deadspace in our anaesthetized sheep preparation where the upper respiratory tract is bypassed by the endotracheal tube. Given the uncertainty that exists in correcting for anatomic deadspace in human studies (American Thoracic Society, 1987) an arbitrary correction constant of 0.5 was applied to Stahl's anatomic deadspace power formula to account for deadspace overestimation as a result of the endotracheal tube bypass of the nasopharynx. The following formula was therefore used to compute anatomic deadspace in the sheep:

$$V_{D,an}\ (ml) = 0.5 \cdot (2.76 \cdot BW^{0.96})$$

Instrumental deadspace

The instrumental deadspace comprised the Fleisch pneumotachograph assembly, valve, endotracheal tube and sample bag deadspace. Deadspace calculated from measurements of the internal dimensions of the equipment was estimated as $100cm^3$.

Appendix 2.3 Method of calculation of the time of breathhold during $T_{L,CO,'sb'}$ measurements. Time of breathholding was calculated according to the recommendations of the Epidemiology Standardization Project (ESP)(Ferris, 1978). The time of breathholding begins when half of the inspired syringe volume has been inhaled and ends when sample collection begins.



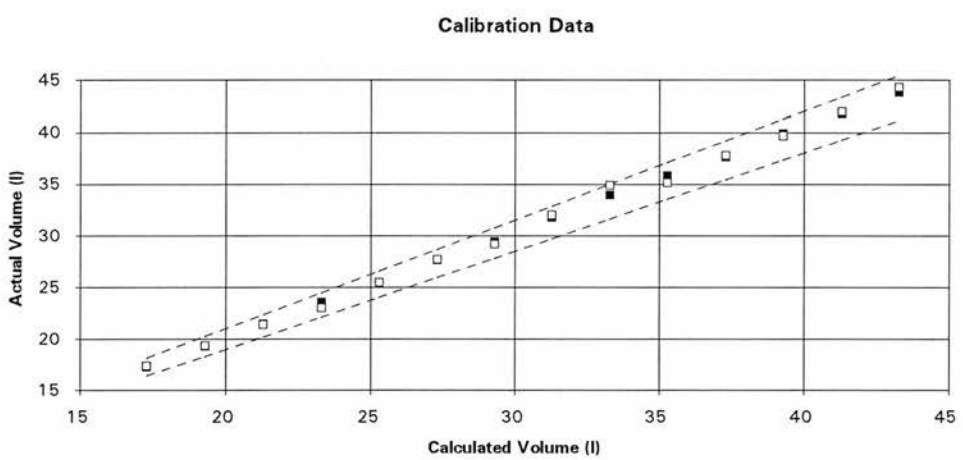
Appendix 2.4 Adjustment of transfer factor measurements for haemoglobin (Hb) concentration.

The following equation was used (Cotes, 1979):

$$T_{L,CO,'sb'} = T_{L,obs} \cdot (a + \theta s \cdot [Hb]) / ((a + \theta s)[Hb])$$

where θs is the reaction rate of carbon monoxide with oxyhaemoglobin at a haemoglobin concentration of 146 g/l and an oxygen tension of 14.7 kPa (335 in SI units), $T_{L,obs}$ is the transfer factor at the subjects own haemoglobin concentration, a is the ratio of the diffusing capacity of the alveolar membrane to the volume of blood in the alveolar capillaries (230 in SI units) and [Hb] is the haemoglobin concentration as a fraction of normal. Use of this equation makes the assumption that the reaction rates of sheep and human haemoglobin with carbon monoxide are similar.

Appendix 2.5 The linearities of the helium and carbon monoxide meters were assessed using the following technique which represents a slight modification of the technique described by Quanjer *et al.*, (1983). Using the reference mixture of carbon monoxide and helium in air (14% helium, 0.3% carbon monoxide, 85.7% air; BOC) a 25 l Douglas bag was repeatedly filled, mixed and then emptied to ensure that the gas mixture in the bag was as near to the reference mixture as possible. Using room air as the baseline, the gain and offset controls of the respective meters were adjusted such that the readings obtained reflected the reference gas concentrations. Most of the gas in the bag was then flushed out by positive pressure. A known volume of room air was then added to the bag using the calibrated syringe, the bag was mixed and the new concentrations of carbon monoxide and helium measured. The volume of the bag was calculated from the new gas concentrations, the known volume of added air and the original gas concentrations. This procedure is repeated until the bag is filled. Linearity was considered acceptable if recorded and calculated volumes agreed within 5% and were linearly related with a regression coefficient of 1.00. The chart below shows calibration data calculated in the above manner, the open and filled squares representing volumes calculated on the basis of carbon monoxide and helium concentrations respectively and the dotted lines representing the upper and lower 5% limits. The respective linear regression coefficients were 1.031 and 1.021.



Appendix 2.6 Results of Cst, $V_{A,eff}$, $T_{L,CO,'sb'}$ and $T_{L/VA}$ measurements made at monthly intervals in nine sheep.

	Cst (l/kPa)				
Sheep No.	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
CON038	1.25	1.90	1.39	1.07	1.44
CON040	2.08	3.14	2.96	3.11	0.83
CON043	1.95	2.10	2.04	1.78	1.51
CON088	1.42	1.61	1.20	1.24	1.57
CON089	1.92	2.29	2.37	2.72	3.01
CON090	0.82	2.20	2.31	1.51	1.92
CON096	1.64	1.79	1.39	1.44	0.97
CON097	1.42	1.62	1.60	1.56	1.41
CON099	1.15	1.56	1.08	1.17	0.93

	Vaeff (l)				
Sheep No.	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
CON038	3.98	4.33	4.40	4.36	4.54
CON040	4.89	4.55	5.02	5.05	4.19
CON043	3.75	3.70	3.85	3.79	3.72
CON088	4.06	3.86	3.55	3.88	4.15
CON089	4.61	4.48	4.69	4.43	4.70
CON090	4.12	4.00	4.06	4.29	4.25
CON096	4.05	3.84	4.00	3.91	3.96
CON097	4.68	4.42	4.91	4.74	4.84
CON099	4.11	3.74	3.90	4.01	4.06

	$T_{L,CO,'sb'}$ (mmol/min/kPa)				
Sheep No.	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
CON038	10.30	10.82	10.05	13.02	12.21
CON040	12.70	12.98	12.24	11.45	11.78
CON043	8.28	8.99	8.09	7.60	7.14
CON088	8.91	8.48	7.80	9.13	9.28
CON089	13.00	10.45	13.53	12.92	13.50
CON090	9.02	6.97	7.32	11.55	10.53
CON096	13.04	10.44	11.86	11.40	13.06
CON097	9.35	7.27	6.41	8.75	9.73
CON099	12.15	11.06	8.54	10.50	9.50

	$T_{L/VA}$ (mmol/min/kPa/l)				
Sheep No.	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
CON038	2.59	2.50	2.29	2.99	2.69
CON040	2.60	2.85	2.44	2.27	2.81
CON043	2.21	2.43	2.10	2.01	1.92
CON088	2.19	2.20	2.20	2.35	2.24
CON089	2.82	2.33	2.88	2.91	2.87
CON090	2.19	1.74	1.80	2.69	2.47
CON096	3.22	2.72	2.96	2.91	3.30
CON097	2.00	1.64	1.31	1.85	2.01
CON099	2.95	2.96	2.19	2.62	2.34

Appendix 2.7 Results of Kolmogorov-Smirnov tests used to test for normal distribution of the pooled and standardized error terms in each anova. The Kolmogorov-Smirnov test statistic *D*, is the absolute value of the largest difference between observed and expected cumulative frequency distributions of the error terms. P=0.01 is the probability that a given value of *D* equals or exceeds the nominal critical value shown when the observed frequency distribution is normally distributed. Since the test statistics were below the nominal critical values in each instance, the null hypothesis, that the error terms were normally distributed, was accepted.

	Standardized error terms			
	Vaeff	Cst	$T_{L,CO,'sb'}$	$T_{L/VA}$
Test Statistic *D*	0.140	0.126	0.117	0.063
Nominal critical value of *D* (p = 0.01)	0.154	0.154	0.154	0.154
Normal distribution (Y/N)	Y	Y	Y	Y

Appendix 2.8 Results of Bartlett's tests of homogeneity of variances applied to the reproducibility data sets used for analysis of variance. The variances of the measurements made on different days were equal.

		Vaeff	Cst	$T_{L,CO,'sb'}$	$T_{L/VA}$
Number of groups	n	5	5	5	5
Sum of variances	Σs^2	0.743	1.574	171.130	7.987
Sum of logged variances	Σ Log s^2	-4.236	-2.668	7.624	0.991
Mean variance	S^2	0.149	0.315	34.226	1.597
Log of mean variance	Log S^2	-0.828	-0.502	1.534	0.203
	d = n . Log S^2 - Σ Log s^2	0.097	0.158	0.048	0.026
Chi square	(X^2) = 2.3026 . (k-1)(d)	1.79	2.92	0.88	0.48
Correction denominator	(D)	1.05	1.05	1.05	1.05
Corrected X^2	-	1.70	2.78	0.84	0.46
Degrees of freedom	d.f.	4	4	4	4
Tabular X^2 (p=0.05)	-	9.49	9.49	9.49	9.49
Homogeneity of variances?	Y/N	Y	Y	Y	Y

Appendix 2.9 Results of analysis of variance for repeated Cst, $V_{A,eff}$, $T_{L,CO,'sb'}$ and $T_{L/VA}$ measurements made in 9 sheep at monthly intervals over a period of 5 months. For all variables there was no significant differences between days. Differences between sheep were highly significant. (NS non-significant, $p>0.05$; *** $p<0.001$).

Static Compliance

Source of variation	d.f.	SS	s^2	F-ratio	Significance
Between days	4	1.683	0.42	2.32	NS
Between sheep	8	8.365	1.05	5.77	***
Within	32	5.800	0.18		
Total	44	15.848			

Effective alveolar volume

Source of variation	d.f.	SS	s^2	F-ratio	Significance
Between days	4	0.186	0.05	1.33	NS
Between sheep	8	5.571	0.70	19.94	***
Within	32	1.118	0.03		
Total	44	6.876			

Transfer factor for carbon monoxide

Source of variation	d.f.	SS	s^2	F-ratio	Significance
Between days	4	119.235	29.81	2.60	NS
Between sheep	8	1172.738	146.59	12.77	***
Within	32	367.431	11.48		
Total	44	1659.404			

Transfer factor expressed per litre of alveolar volume

Source of variation	d.f.	SS	s^2	F-ratio	Significance
Between days	4	5.043	1.26	2.15	NS
Between sheep	8	53.106	6.64	11.31	***
Within	32	18.775	0.59		
Total	44	76.924			

Appendix 2.10 Results of Cst, $V_{A,eff}$, $T_{L,CO,'sb'}$ and $T_{L/VA}$ measurements made in sheep not included in the reproducibility analysis (Appendix 2.2).

		BW	Cst	$V_{A,eff}$	$T_{L,CO,'sb'}$	$T_{L/VA}$
Sheep No	Day	(kg)	(l/kPa)	(l)	(mmol/min/kPa)	(mmol/min/kPa/l)
CON042	0	70	2.65	4.56	10.07	2.21
CON042	47	62	2.68	4.45	9.78	2.20
CON042	78	62	2.37	4.68	11.11	2.37
CON042	110	60	2.06	4.69	10.88	2.32
CON091	0	82	1.89	4.26	11.49	2.69
CON091	35	82	1.63	4.20	10.84	2.58
CON091	82	75	1.77	4.09	10.51	2.57
CON091	114	75	1.60	4.17	11.58	2.78
CON091	146	74	0.86	2.43	6.07	2.50
CON092	0	85	2.23	4.71	11.84	2.51
CON092	49	77	2.28	4.51	9.51	2.11
CON092	79	78	1.94	4.72	13.06	2.77
CON093	0	63	1.73	4.28	8.43	1.97
CON093	51	63	2.15	4.42	9.68	2.19
CON093	81	64	2.08	4.90	11.58	2.36
CON094	0	52	1.33	3.33	9.25	2.77
CON094	51	54	1.45	3.56	10.45	2.93
CON094	81	54	1.40	3.58	10.70	2.99
CON094	112	53	1.26	3.61	10.68	2.96
CON095	0	62	2.24	4.74	9.06	1.91
CON095	50	64	1.38	4.65	9.76	2.10
CON095	81	63	2.21	4.64	10.64	2.29
CON098	0	87	1.58	4.90	13.56	2.77
CON098	37	87	2.14	4.58	13.79	3.01
CON098	114	85	1.51	4.78	13.22	2.77

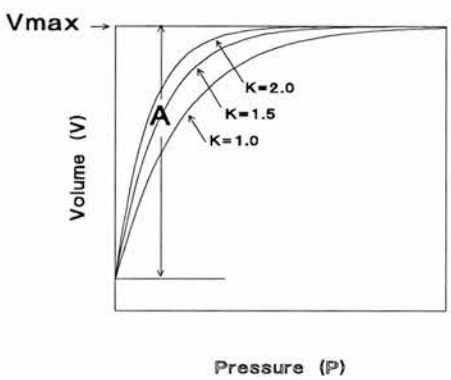
Appendix 3.1 Sheep used to provide data for exponential analysis of lung pressure-volume characteristics. All sheep as described in appendix 2.1 were utilised (with the exception of CON098). In addition, the following 3 Oxford x Texel cross sheep were included in this study.

Sheep No.	Bodyweight (kg)	Breed	Sex	Year of Birth
CON101	82	Oxford x Texel	F	1988
CON103	78	Oxford x Texel	F	1988
CON105	82	Oxford x Texel	F	1988

Appendix 3.2 Method used in exponential curve-fitting analysis of pressure-volume curves.

The underlying hypothesis is that pressure-volume curves in sheep fit a function expressed by

$$V = Vmax - A \cdot e^{-K \cdot P}$$



where P is the static recoil pressure, Vmax represents the volume asymptote, A is the difference between Vmax and the intercept on the volume axis and *K* represents the slope of the P-V curve and therefore defines its shape.

The above equation can be transformed logarithmically to:

$$\ln (1-V/Vmax) = A - K \cdot P$$

which has the form of a straight line. Least squares regression analysis is then used to find the slope and intercept of this line. Because, Vmax is unknown at the outset, it is necessary to repeat the computation with different values of Vmax until the best fit is achieved as adjudged by a maximal value of the coefficient of determination (r^2). This value of Vmax is then used in the regression and values of A and *K* derived. The iterative computations were executed on a personal computer (IBM PS/2) using the spreadsheet facility of a commercial software package (Microsoft® Works; Microsoft Corporation, Redmond, WA).

Appendix 3.3 Results of curve fitting analysis over the whole pressure-volume range for 18 sheep.

	Cst	$V_{A,eff}$	r^2	Vmax	A	K
Sheep No	(l/KPa)	(l)	(%)	(ml)	(ml)	-
CON043	2.043	3.85	98.87	3743	1804	1.815
CON090	2.305	4.06	99.09	3932	2487	1.587
CON042	2.686	4.45	99.65	4213	2805	2.063
CON040	2.960	5.02	97.98	4755	3034	2.119
CON092	2.285	4.51	98.58	4536	2567	1.399
CON091	1.770	4.09	98.21	4055	2467	1.287
CON095	1.376	4.65	98.86	4450	2366	1.519
CON099	1.080	3.90	98.63	3859	2293	1.091
CON105	2.018	4.30	98.91	4123	2948	1.826
CON103	1.998	4.42	98.57	4312	3252	1.426
CON101	1.958	3.94	96.24	3663	2552	1.746
CON038	1.393	4.40	97.19	4337	2583	1.181
CON096	1.388	4.00	99.74	3912	2532	1.434
CON089	2.372	4.69	99.47	4508	2777	1.586
CON093	2.151	4.42	99.07	4374	2975	1.477
CON097	1.604	4.91	98.89	4896	2368	1.155
CON088	1.199	3.55	98.57	3277	1590	1.416
CON094	1.454	3.56	96.11	3470	1920	1.345

Appendix 3.4.1 Results of sign tests as applied to exponential curves (Appendix 3.3) fitted to the pressure-volume data.

Sheep No.	No. of data points	BELOW	EQUAL	ABOVE	P-VALUE	MEDIAN	PASS? (P>0.05)
CON043	27	16	0	11	0.4421	-6.993	Y
CON090	40	21	0	19	0.8746	-7.547	Y
CON042	38	26	0	12	0.0336	-12.84	N
CON040	30	13	0	17	0.5847	7.657	Y
CON092	45	16	0	29	0.0725	35.31	Y
CON091	41	18	0	23	0.5327	13.98	Y
CON095	43	22	0	21	1.0000	-0.8826	Y
CON099	37	21	0	16	0.5114	-5.638	Y
CON105	43	23	0	20	0.7608	-2.620	Y
CON103	43	18	0	25	0.3604	20.06	Y
CON101	43	17	0	26	0.2221	22.03	Y
CON038	39	23	0	16	0.3368	-13.59	Y
CON096	25	12	0	13	1.0000	0.8086	Y
CON089	47	26	0	21	0.5601	-16.15	Y
CON093	44	21	0	23	0.8804	6.217	Y
CON097	39	20	0	19	1.0000	-4.445	Y
CON088	25	12	0	13	1.0000	3.622	Y
CON094	40	17	0	23	0.4296	14.49	Y

Appendix 3.4.2 Results of runs tests as applied to exponential curves (Appendix 3.3) fitted to the pressure-volume data.

Sheep No.	Observed No. of runs	Expected No. of runs	Significance (P)	PASS?
CON043	6	14.0370	0.0011	N
CON090	10	20.9500	0.0005	N
CON042	13	17.4211	0.0914	Y
CON040	4	15.7333	0.0000	N
CON092	8	21.6222	0.0000	N
CON091	6	21.1951	0.0000	N
CON095	7	22.4884	0.0000	N
CON099	10	19.1622	0.0019	N
CON105	7	22.3953	0.0000	N
CON103	5	21.9302	0.0000	N
CON101	8	21.5581	0.0000	N
CON038	4	19.8718	0.0000	N
CON096	11	13.4800	0.3103	Y
CON089	10	24.2340	0.0000	N
CON093	12	22.9545	0.0008	N
CON097	8	20.4872	0.0001	N
CON088	5	13.4800	0.0005	N
CON094	9	20.5500	0.0002	N

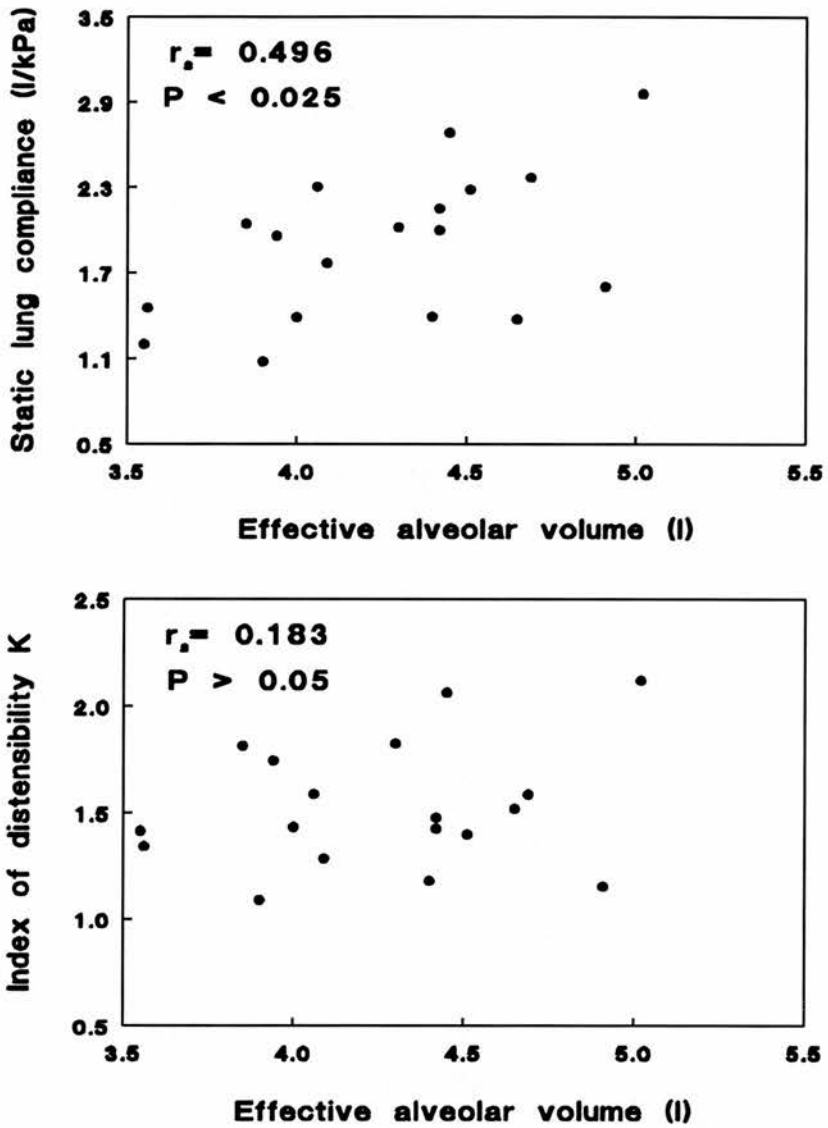
Appendix 3.5 Results of curve fitting analysis over the restricted pressure-volume range for 18 sheep (only points greater than 50% TLC used).

	r^2	Vmax	A	K
Sheep No	(%)	(ml)	(ml)	-
CON043	98.32	3704	1819	1.988
CON090	98.49	4000	2285	1.329
CON042	99.22	4350	2461	1.512
CON040	97.66	4756	2990	2.098
CON092	97.83	4504	2468	1.362
CON091	97.89	4049	2519	1.326
CON095	97.84	4619	2348	1.161
CON099	98.63	4030	2215	0.820
CON105	99.39	4286	2676	1.344
CON103	98.35	4313	3205	1.416
CON101	95.49	3810	2318	1.296
CON038	97.13	4349	2542	1.142
CON096	99.14	4042	2394	1.119
CON089	99.39	4513	2714	1.551
CON093	98.88	4384	2887	1.430
CON097	98.89	4896	2368	1.155
CON088	98.12	3556	1724	0.872
CON094	95.42	3468	1902	1.349

Appendix 3.6. Summary results from exponential pressure-volume curve fitting analysis using the restricted data range (only points greater than 50% TLC used).

Measurement	Median	Range
Data points/animal	37	25 - 43
K	1.337	0.820 - 2.098
A (ml)	2428	1724 - 3205
Vmax (ml)	4300	3468 - 4896
r^2	98.34	95.42 - 99.39
Cst (l/kPa)	2.050	1.131 - 3.698

Appendix 3.7 Graphs demonstrating the relationship between *K* and Cst and $V_{A,eff}$. As expected there was a significant positive relationship between Cst and $V_{A,eff}$. However no significant relationship could be demonstrated between *K* and $V_{A,eff}$.



Appendix 3.8 The Spearman rank-order correlation coefficient was used to determine whether any significant correlation existed between *K* and age.

N		18
Sum of squared differences	$Sumd^2$	1330
r_s	$1 - [(6 \times Sumd^2)/(N^3-N)]$	-0.373

The correlation coefficient was not significant (P>0.05).

Appendix 3.9 Results of exponential curve analysis on pressure-volume curves measured at monthly intervals in six sheep.

			K		
Sheep No.	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
CON038	1.087	1.054	1.587	0.986	0.995
CON043	2.005	1.530	1.181	1.145	1.727
CON088	1.006	1.541	0.820	1.194	1.725
CON090	1.118	1.719	2.092	2.446	0.768
CON097	0.984	1.180	1.348	1.130	1.232
CON099	1.100	1.162	1.155	1.029	1.257

			Vmax (ml)		
Sheep No.	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
CON038	4280	4013	3932	4054	4602
CON043	3563	4136	4337	4290	3489
CON088	4151	3887	4030	3963	3836
CON090	4204	3596	3700	3536	4201
CON097	4755	4421	3295	4264	4082
CON099	4104	3750	4896	4830	4819

			A (ml)		
Sheep No.	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
CON038	2597	2537	2487	2048	2882
CON043	2138	2684	2583	2844	2015
CON088	2577	2297	2215	2210	2643
CON090	2725	1865	1883	1896	2056
CON097	2403	2234	1607	2200	2403
CON099	1884	2207	2368	2511	2827

Appendix 3.9 (contd.) Results of exponential curve analysis on pressure-volume curves measured at monthly intervals in six sheep.

	r^2				
Sheep No.	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
CON038	98.86	99.18	99.09	98.54	99.21
CON043	98.97	96.60	97.19	99.66	99.86
CON088	98.84	98.05	98.66	99.78	99.28
CON090	98.53	99.38	98.30	97.38	98.95
CON097	99.21	98.80	98.75	98.80	94.34
CON099	98.45	99.27	98.89	99.10	98.00

Appendix 3.10 Reproducibility of exponential curve analysis on pressure-volume curves measured at monthly intervals in six sheep. Only curve fits in which r^2 exceeded 98% were used in the computation of these statistics. The coefficient of variation (CV) statistics refer to summary statistics of repeated measurements on the six sheep i.e. for *K*, the mean CV for repeated measurements between the six sheep was 23.13%. The results indicate that *K* measurements are poorly reproducible over time.

	Variable			
CV (%)	r^2	Vmax	A	*K*
μ	0.37	7.65	12.91	23.13
Median	0.40	8.35	13.80	20.90
Range	0.19 - 0.59	2.78 - 12.98	7.73 - 16.43	6.60 - 36.10

Appendix 4.1 Details of MVV-infected sheep used to provide lung function data (Chapter 4, section 4.2.1) and establish the spectrum of pulmonary dysfunction present within a flock of naturally infected sheep. The results of the agar gel immunodiffusion test (Winward *et al.*, 1979) are given together with the test date (-ve = no reaction; +ve = reaction; inc. = inconclusive result; * = not done).

		Test Date					
Sheep No.	Year of Birth	17/8/90	20/11/90	19/12/90	25/1/91	18/6/91	17/6/92
MVV08N	1985	*	*	*	*	+ve	*
MVV001	1988	-ve	inc.	-ve	+ve	*	*
MVV002	1988	-ve	-ve	+ve	+ve	+ve	*
MVV006	1986	+ve	+ve	+ve	+ve	+ve	+ve
MVV007	1987	+ve	+ve	+ve	+ve	+ve	+ve
MVV009	1985	+ve	+ve	-ve	+ve	+ve	*
MVV010	1985	+ve	+ve	-ve	+ve	+ve	inc.
MVV011	1986	+ve	+ve	+ve	+ve	+ve	+ve
MVV012	1985	+ve	+ve	+ve	+ve	+ve	*
MVV013	1987	+ve	+ve	+ve	+ve	+ve	+ve
MVV017	1987	+ve	+ve	+ve	+ve	+ve	+ve
MVV021	1987	+ve	+ve	+ve	+ve	+ve	+ve
MVV025	1986	+ve	+ve	+ve	+ve	*	+ve
MVV026	1987	+ve	inc.	-ve	+ve	-ve	+ve
MVV029	1986	+ve	+ve	+ve	+ve	+ve	*
MVV032	1986	+ve	+ve	+ve	+ve	*	+ve
MVV033	1985	+ve	+ve	+ve	+ve	+ve	*
MVV035	1987	+ve	+ve	+ve	+ve	+ve	+ve
MVV039	1986	+ve	+ve	+ve	+ve	*	+ve
MVV040	1985	+ve	+ve	+ve	+ve	+ve	*
MVV045	1987	+ve	+ve	+ve	+ve	-ve	inc.
MVV047	1986	+ve	+ve	+ve	+ve	+ve	*
MVV053	1986	+ve	+ve	+ve	+ve	-ve	*
MVV057	1985	-ve	*	+ve	+ve	-ve	-ve
MVV059	1985	+ve	+ve	-ve	+ve	+ve	inc.
MVV064	1986	+ve	+ve	+ve	+ve	+ve	+ve
MVV065	1986	+ve	+ve	inc.	+ve	+ve	+ve
MVV068	1986	+ve	+ve	+ve	+ve	+ve	*
MVV070	1985	inc.	+ve	-ve	inc.	-ve	-ve
MVV071	1986	+ve	-ve	+ve	+ve	+ve	+ve
MVV073	1986	+ve	+ve	+ve	+ve	+ve	+ve
MVV074	1986	+ve	+ve	+ve	+ve	+ve	*
MVV075	1986	+ve	+ve	+ve	+ve	+ve	+ve
MVV076	1987	+ve	+ve	+ve	+ve	+ve	+ve
MVV078	1986	-ve	+ve	+ve	+ve	+ve	-ve
MVV079	1987	+ve	inc.	-ve	inc.	*	*
MVV080	1985	+ve	+ve	-ve	+ve	+ve	+ve
MVV083	1986	*	-ve	+ve	inc.	-ve	*
MVV089	1985	*	+ve	+ve	+ve	+ve	*
MVV090	1986	*	+ve	+ve	+ve	+ve	*
MVV091	1985	*	+ve	+ve	+ve	-ve	*

Appendix 4.1 contd. Details of MVV-infected sheep used to provide lung function data (Chapter 4, section 4.2.1) and establish the spectrum of pulmonary dysfunction present within a flock of naturally infected sheep.

		Test Date					
Sheep No.	Year of Birth	17/8/90	20/11/90	19/12/90	25/1/91	18/6/91	17/6/92
MVV092	1986	*	+ve	+ve	+ve	+ve	*
MVV093	1985	*	+ve	+ve	+ve	+ve	*
MVV096	1986	*	+ve	+ve	+ve	+ve	*
MVV097	1986	*	-ve	+ve	-ve	-ve	*
MVV099	1986	+ve	+ve	+ve	+ve	+ve	+ve
MVV100	1986	-ve	-ve	+ve	-ve	-ve	-ve
MVV105	1986	*	-ve	-ve	+ve	-ve	-ve
MVV116	1986	+ve	+ve	+ve	+ve	+ve	+ve
MVV118	1987	+ve	+ve	+ve	+ve	*	+ve
MVV130	1986	+ve	+ve	+ve	+ve	-ve	inc.

Appendix 4.2 Semi-quantitative clinical scoring procedure used to objectively assess respiratory disease in MVV-infected sheep. Clinical details were recorded on a worksheet (Appendix 4.2.1 & 4.2.2) and the following arbitrary scores added according to the presence or absence of particular clinical criterion.

Presence of:	Score
Abnormal respiratory character	20
Dyspnoea	100
Mouth breathing	100
Cough	50
Nasal discharge	25
Auscultation - increased volume	25
Auscultation - adventitious sounds	50

CLINICAL RECORD WORKSHEET DATE BREED:

Respiratory rate: Character: Normal ( ) Shallow ( ) Deep ( )

Presence of dyspnoea: No ( ) Yes ( ) Insp. ( ) Exp. ( ) Inc. ( )

Presence of Mouth Breathing: No ( ) Yes ( ) Inter. ( ) All ( )

Presence of Cough: No ( ) Yes ( ) Type: Deep ( ) Dry ( ) Shallow ( ) Moist ( )

Posture normal: Yes ( ) No ( ) Comment:

Head posture normal: Yes ( ) No ( ) Comment:

Demeanour: Normal ( ) Dull ( ) Very Depressed ( )

Appetite: Normal ( ) Inappetent ( ) Anorexic ( ) Unknown ( )

Gait normal: Yes ( ) No ( )

(1-10)

	LF	LH	RF	RH
Paresis				
Ataxia				
Spasticity				
Hypometria				
Hypermetria				
Total Deficit				
Other				

Lameness present: No ( ) Yes ( )

Visible Jt. Swellings: No ( ) Yes ( )

Faeces normal: Yes ( ) No ( ) Comment:

Yellow Ear tag No.: Other Ear tag Nos.:

Body Temperature: Condition score:

Anal tone normal: yes ( ) no ( ) comment:

Discharges: Nasal No ( ) Yes ( ) Ocular No ( ) Yes ( )

	R	L	Amount (1-2)
Character Ser.			
SM.			
Muc.			
MP.			
Food.			

	R	L	Amount (1-2)
Character Ser.			
SM.			
Muc.			
MP.			
Food.			

Presence of Normal:-

Face Symmetry: Yes ( ) No ( ) Comment:

Eye Symmetry: Yes ( ) No ( ) Comment:

Spontaneous nystagmus: No ( ) Yes ( ) Comment:

Vestibular nystagmus: Yes ( ) No ( ) Comment:

Eye Movements - Head raised: Yes ( ) No ( ) Comment:

Head flexed: Yes ( ) No ( ) Comment:

Clockwise rotation: Yes ( ) No ( ) Comment:

Anticlockwise rotation: Yes ( ) No ( ) Comment:

Corneal Reflex: Yes ( ) No ( ) Comment:

Menace Reflex: Yes ( ) No ( ) Comment:

Sens. Blink Reflex: Yes ( ) No ( )

RL	RM	LL	LM

Comment:

Pupil Sizes: Yes ( ) No ( ) Comment:

Pupillary Light Reflex: Yes ( ) No ( ) Comment:

Ocular Mucous Membranes: Yes ( ) No ( ) Comment: R

L

Adenitis: No ( ) Yes ( ) SM ( ) PS ( ) PF ( ) (1-2)

Jaw Tone: Yes ( ) No ( ) Comment:

Normal oral cavity: Yes ( ) No ( ) Comment:

Teeth: No. of Permanents: Broken Mouthed: No ( ) Yes ( )

No. of Temporaries:

Induced cough: No ( ) Yes ( ) Type: Deep ( ) Dry ( ) Shallow ( ) Moist ( )

Heart Rate:

Weight:

Mammary Glands Normal: Yes ( ) No ( ) Comment: R

L

Lactating: No ( ) Yes ( ) Milk Normal: Yes ( )
No ( ) Comment:

JOINTS

	LF	LH	RF	RH	Comment
Palpable swellings No ( ) Yes ( )					
Crepitus No ( ) Yes ( )					

FEET

Normal Appearance Yes ( ) No ( )	LF	LH	RF	RH

Foot Rot: No ( ) Yes ( )									
Scald: No ( ) Yes ( )									
Overgrown: No ( ) Yes ( )									
Infected: No ( ) Yes ( )									
Other:									
Even Horn Wear Yes ( ) No ( )	L	M	L	M	L	M	L	M	(>or<)

Tilted: No ( ) Yes ( ) Comment:

Normal Righting Reflex

RHS: Yes ( ) No ( ) Comment:

LHS: Yes ( ) No ( ) Comment:

Auscultation

	Right				Left			
Vol.	Dorsal	Mid	Ventral	TR.	Dorsal	Mid	Ventral	Comment
3+								
2+								
1+								
0								
1-								
2-								
3-								
Adv.								
CR.I								
CR.E								
WH.I								
WH.E								

Rumen Motility (3mins)

No. of Primary Contractions:

No. of Secondary contractions:

Knuckling Present: No ( ) Yes ( )	RF	RH	LF	LH	(1-3+sec)

Normal CV Ausc.: Yes ( ) No ( ) Comment:

Thoracic Perimeter:

Sheep No	Date of measurement	Age	BW	Clinical Score	Observed Cst	Predicted Cst	Observed $V_{A,eff}$	Predicted $V_{A,eff}$	Observed $T_{L,CO,'sb'}$	Predicted $T_{L,CO,'sb'}$	Observed $T_{L/VA}$	Predicted $T_{L/VA}$
		(yrs)	(Kgs)	-	(l/kPa)	(l/kPa)	(l)	(l)	(mmol/min /kPa)	(mmol/min /kPa)	(mmol/min /kPa/l)	(mmol/mi /kPa/l)
MVV08N	9/10/91	6.61	50	0	1.876	1.465	4.78	4.00	ND	8.77	ND	2.24
MVV08N	22/11/91	6.73	50	ND	2.205	1.465	4.52	4.00	9.44	8.77	2.09	2.24
MVV001	9/10/91	3.61	51	0	1.280	1.485	3.16	4.01	ND	8.88	ND	2.25
MVV001	19/11/91	3.72	51	ND	1.756	1.485	3.37	4.01	7.43	8.88	2.21	2.25
MVV002	28/11/91	3.75	56	0	0.815	1.586	2.45	4.10	4.83	9.43	1.97	2.32
MVV002	5/3/92	4.01	46	20	0.556	1.385	2.03	3.92	2.15	8.33	1.06	2.18
MVV006	5/5/93	7.18	54	ND	1.173	1.545	4.49	4.07	9.14	9.21	2.04	2.29
MVV009	17/10/91	6.63	40	270	0.554	1.264	2.85	3.81	3.76	7.67	1.32	2.09
MVV010	15/7/92	7.38	54	ND	2.175	1.545	5.05	4.07	9.15	9.21	1.81	2.29
MVV010	18/9/92	7.56	53	ND	2.348	1.525	4.98	4.05	8.47	9.10	1.70	2.28
MVV010	29/4/93	8.17	50	ND	1.476	1.465	4.85	4.00	7.89	8.77	1.63	2.24
MVV011	5/5/93	7.18	68	ND	1.176	1.827	4.20	4.32	10.74	10.75	2.56	2.49
MVV013	15/7/92	5.38	54	ND	1.446	1.545	4.47	4.07	6.95	9.21	1.56	2.29
MVV013	18/9/92	5.56	58	ND	1.855	1.626	4.37	4.14	7.10	9.65	1.62	2.35
MVV013	29/4/93	6.17	60	ND	0.995	1.666	3.70	4.18	5.29	9.87	1.43	2.38
MVV021	6/5/93	6.19	67	ND	1.245	1.807	4.24	4.30	12.73	10.64	3.00	2.48
MVV025	5/5/93	8.18	54	ND	1.412	1.545	3.72	4.07	8.60	9.21	2.31	2.29
MVV026	7/5/93	6.19	76	ND	2.380	1.988	4.07	4.47	10.97	11.63	2.70	2.61
MVV029	26/11/91	5.74	54	95	2.136	1.545	3.60	4.07	6.73	9.21	1.87	2.29
MVV029	4/3/92	6.01	49	ND	1.575	1.445	3.79	3.98	4.20	8.66	1.11	2.22

Appendix 4.3 Results of Cst, $V_{A,eff}$, $T_{L,CO,'sb'}$ and $T_{L/VA}$ measurements made in 51 sheep naturally infected with MVV. Ages shown are the ages at the time of lung function measurements. The year, but not the actual birth date, were known for the sheep, therefore a uniform birthday of the 1st of March was assumed for each animal in the calculation of ages. Predicted values for control animals of the same bodyweight are also given.

Sheep No	Date of measurement	Age	BW	Clinical Score	Observed Cst	Predicted Cst	Observed $V_{A,eff}$	Predicted $V_{A,eff}$	Observed $T_{L,CO,'sb'}$	Predicted $T_{L,CO,'sb'}$	Observed $T_{L/VA}$	Predicted $T_{L/VA}$
		(yrs)	(Kgs)	-	(l/kPa)	(l/kPa)	(l)	(l)	(mmol/min /kPa)	(mmol/min /kPa)	(mmol/min /kPa/l)	(mmol/min /kPa/l)
MVV033	11/6/92	7.28	57	ND	2.141	1.606	4.18	4.12	10.54	9.54	2.52	2.34
MVV035	6/5/93	6.19	59	ND	1.089	1.646	3.23	4.16	6.02	9.76	1.86	2.36
MVV039	7/5/93	7.19	66	ND	2.074	1.787	5.16	4.28	11.20	10.53	2.17	2.46
MVV040	3/10/91	6.59	58	0	1.226	1.626	4.47	4.14	9.47	9.65	2.12	2.35
MVV45	6/5/93	6.19	57	ND	2.037	1.606	4.05	4.12	10.20	9.54	2.52	2.34
MVV047	10/10/91	5.61	45	125	1.036	1.365	2.64	3.90	6.77	8.22	2.56	2.16
MVV047	20/11/91	5.73	45	ND	0.268	1.365	1.44	3.90	4.89	8.22	3.39	2.16
MVV047	31/1/92	5.92	42	ND	0.584	1.304	1.87	3.85	2.65	7.89	1.42	2.12
MVV057	15/6/92	7.30	59	ND	2.564	1.646	5.09	4.16	8.03	9.76	1.58	2.36
MVV057	30/4/93	8.17	63	ND	1.567	1.726	4.65	4.23	10.28	10.20	2.21	2.42
MVV064	5/5/93	7.18	50	ND	1.039	1.465	3.62	4.00	9.75	8.77	2.69	2.24
MVV065	26/11/91	5.74	75	25	1.865	1.968	3.52	4.45	10.55	11.52	2.99	2.59
MVV065	4/3/92	6.01	74	ND	2.149	1.947	4.16	4.43	10.49	11.41	2.52	2.58
MVV070	7/5/93	8.19	56	ND	1.643	1.586	4.51	4.10	12.21	9.43	2.71	2.32
MVV071	7/5/93	7.19	68	ND	1.597	1.827	3.69	4.32	10.09	10.75	2.74	2.49
MVV075	3/10/91	5.59	57	25	1.642	1.606	3.83	4.12	6.73	9.54	1.76	2.34
MVV075	22/11/91	5.73	57	ND	1.258	1.606	3.40	4.12	7.75	9.54	2.28	2.34
MVV075	4/3/92	6.01	66	ND	2.205	1.787	4.09	4.28	6.85	10.53	1.67	2.46
MVV075	6/5/93	7.19	58	ND	2.567	1.626	4.48	4.14	8.83	9.65	1.97	2.35
MVV076	22/11/91	4.73	60	25	0.924	1.666	3.23	4.18	8.59	9.87	2.66	2.38
MVV076	4/3/92	5.01	61	ND	1.284	1.686	3.27	4.19	7.30	9.98	2.23	2.39
MVV076	6/5/93	6.19	66	ND	1.626	1.787	3.85	4.28	9.33	10.53	2.42	2.46
MVV078	6/5/93	7.19	62	ND	1.871	1.706	4.43	4.21	9.16	10.09	2.07	2.41
MVV080	1/10/91	6.59	54	120	1.176	1.545	3.33	4.07	ND	9.21	ND	2.29

Appendix 4.3 contd.

Sheep No	Date of measurement	Age	BW	Clinical Score	Observed Cst	Predicted Cst	Observed $V_{A,eff}$	Predicted $V_{A,eff}$	Observed $T_{L,CO,'sb'}$	Predicted $T_{L,CO,'sb'}$	Observed $T_{L/VA}$	Predicted $T_{L/VA}$
		(yrs)	(Kgs)	-	(l/kPa)	(l/kPa)	(l)	(l)	(mmol/min /kPa)	(mmol/min /kPa)	(mmol/min /kPa/l)	(mmol/min /kPa/l)
MVV080	20/11/91	6.73	54	ND	1.417	1.545	3.33	4.07	6.09	9.21	1.83	2.29
MVV080	5/3/92	7.02	54	75	1.382	1.545	3.26	4.07	7.23	9.21	2.22	2.29
MVV080	26/5/92	7.24	52	ND	1.851	1.505	3.76	4.03	8.67	8.99	2.30	2.26
MVV080	22/4/93	8.15	38	ND	ND	1.224	3.32	3.78	ND	7.45	ND	2.06
MVV083	31/1/92	5.92	70	25	2.353	1.867	5.50	4.36	11.13	10.97	2.02	2.52
MVV091	3/2/92	6.93	80	20	1.275	2.068	3.88	4.54	10.09	12.07	2.60	2.66
MVV099	8/6/92	6.28	50	ND	1.476	1.465	3.65	4.00	4.93	8.77	1.35	2.24
MVV100	30/4/93	7.17	62	ND	1.614	1.706	4.57	4.21	9.01	10.09	1.97	2.41
MVV105	7/5/93	7.19	64	ND	ND	1.746	4.69	4.25	11.73	10.31	2.50	2.44
MVV116	6/5/93	7.19	54	ND	1.671	1.545	4.31	4.07	6.62	9.21	1.54	2.29
MVV118	7/5/93	6.19	64	ND	1.610	1.746	4.01	4.25	10.21	10.31	2.54	2.44
MVV130	7/5/93	7.19	52	ND	1.152	1.505	3.92	4.03	10.86	8.99	2.77	2.26
MVV007	5/8/92	5.44	49	0	1.452	1.445	3.61	3.98	6.42	8.66	1.78	2.22
MVV012	31/1/92	6.92	67	25	2.263	1.807	4.64	4.30	7.39	10.64	1.59	2.48
MVV017	18/6/92	5.30	50	ND	1.554	1.465	4.62	4.00	9.51	8.77	2.06	2.24
MVV032	11/12/92	6.79	55	0	1.671	1.566	3.82	4.09	8.09	9.32	2.12	2.31
MVV053	28/11/91	5.75	57	295	1.607	1.606	4.09	4.12	6.27	9.54	1.53	2.34
MVV053	5/2/92	5.94	50	ND	0.887	1.465	2.97	4.00	2.61	8.77	0.88	2.24
MVV059	1/6/92	7.26	52	ND	1.327	1.505	3.52	4.03	5.53	8.99	1.57	2.26
MVV059	8/7/92	7.36	47	245	1.265	1.405	3.44	3.94	6.43	8.44	1.87	2.19
MVV068	5/3/92	6.02	52	95	1.026	1.505	2.85	4.03	3.12	8.99	1.09	2.26
MVV073	18/9/92	6.56	50	ND	1.116	1.465	4.19	4.00	7.01	8.77	1.67	2.24
MVV074	1/10/91	5.59	55	25	0.954	1.566	3.20	4.09	3.77	9.32	1.18	2.31

Appendix 4.3 contd.

Sheep No	Date of measurement	Age	BW	Clinical Score	Observed Cst	Predicted Cst	Observed $V_{A,eff}$	Predicted $V_{A,eff}$	Observed $T_{L,CO,'sb'}$	Predicted $T_{L,CO,'sb'}$	Observed $T_{L/VA}$	Predicted $T_{L/VA}$
		(yrs)	(Kgs)	-	(l/kPa)	(l/kPa)	(l)	(l)	(mmol/min /kPa)	(mmol/min /kPa)	(mmol/min /kPa/l)	(mmol/min /kPa/l)
MVV074	20/11/91	5.73	55	ND	0.889	1.566	2.70	4.09	2.90	9.32	1.07	2.31
MVV074	5/3/92	6.02	60	270	1.166	1.666	2.69	4.18	2.96	9.87	1.10	2.38
MVV079	3/6/92	5.26	50	ND	0.992	1.465	2.29	4.00	2.75	8.77	1.20	2.24
MVV079	9/9/92	5.53	51	ND	0.690	1.485	1.88	4.01	2.07	8.88	1.10	2.25
MVV089	3/2/92	6.93	65	100	0.672	1.767	3.40	4.27	6.67	10.42	1.96	2.45
MVV090	31/1/92	5.92	67	0	1.581	1.807	4.77	4.30	9.13	10.64	1.91	2.48
MVV092	3/2/92	5.93	64	0	2.705	1.746	4.43	4.25	7.71	10.31	1.74	2.44
MVV093	3/2/92	6.93	62	25	2.189	1.706	4.15	4.21	6.89	10.09	1.66	2.41
MVV096	31/1/92	5.92	62	100	1.482	1.706	3.86	4.21	7.49	10.09	1.94	2.41
MVV097	3/2/92	5.93	60	0	2.406	1.666	4.58	4.18	8.78	9.87	1.92	2.38

Appendix 4.3 contd.

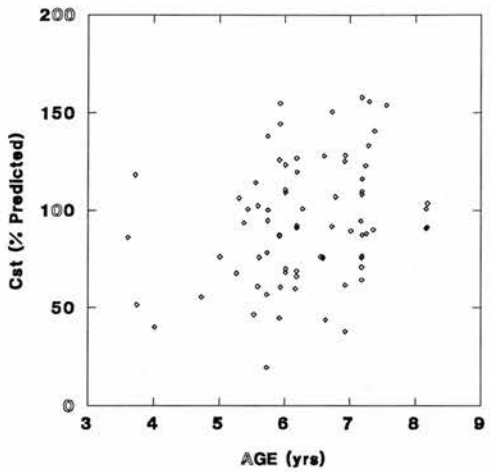
Appendix 4.4 Results of Mann-Whitney U tests applied to the comparison of actual and predicted lung function values for the whole data set and for individual age groups.

Age Group	Statistic	Cst	$V_{A,eff}$	$T_{L,CO,'sb'}$	$T_{L/VA}$
All Data	n	75	77	73	73
	Mann-Whitney U Test Statistic	2336	2318	1383	1384
	P	0.073	0.019	0.000	0.000
4	n	4	4	3	3
	Mann-Whitney U Test Statistic	4	0	0	1
	P	0.245	0.020	0.050	0.127
5	n	6	6	6	6
	Mann-Whitney U Test Statistic	6	12	4	7
	P	0.054	0.336	0.025	0.078
6	n	31	31	31	31
	Mann-Whitney U Test Statistic	397	234	201	264
	P	0.240	0.001	0.000	0.002
7	n	29	30	28	28
	Mann-Whitney U Test Statistic	395	464	255	210
	P	0.691	0.836	0.025	0.003
8	n	5	6	5	5
	Mann-Whitney U Test Statistic	13	24	10	8
	P	0.917	0.337	0.602	0.347

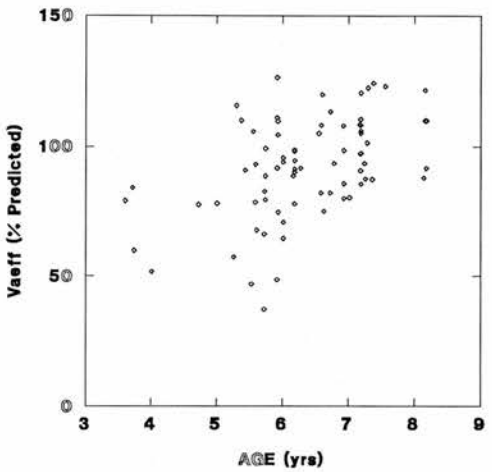
Appendix 4.5 Results of Kruskal-Wallis one-way analysis of variance by ranks. This test was used to determine whether variation in lung function was related to the age group of the sheep. The results indicate that $V_{A,eff}$ and $T_{L,CO,'sb'}$ values in MVV-infected sheep are related to the age group that the animals belong to.

	Cst	$V_{A,eff}$	$T_{L,CO,'sb'}$	$T_{L/VA}$
Kruskal-Wallis Test Statistic	4.442	16.163	15.289	3.345
P	0.350	0.003	0.004	0.502

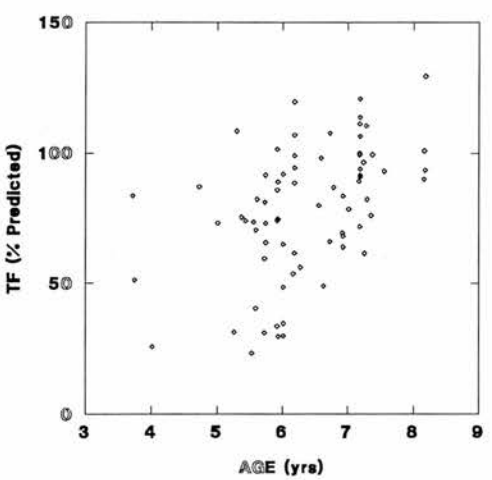
Appendix 4.6.1-4.6.4. The relationship between age and pulmonary dysfunction. The multiple correlation (r) and the significance of the relationship for each variable with age are given. Normal probability plots and plots of studentized residuals against estimated values were used to establish that error terms were normally distributed, had constant variance and were independent (Systat for Windows, v.5; Systat Inc., Evanston, IL, USA).



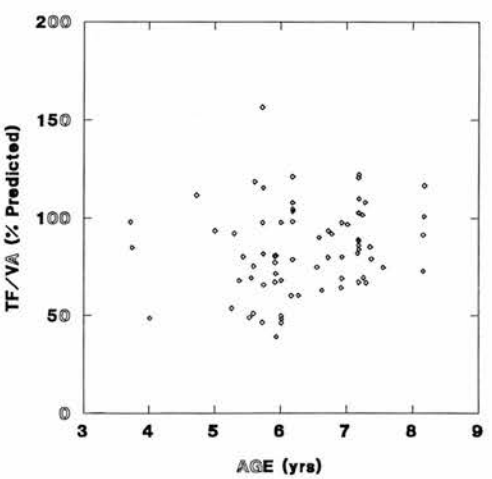
Appendix 4.6.1 Relationship between age and percent predicted Cst. The relationship is significant (r = 0.278;P=0.016).



Appendix 4.6.2 Relationship between age and percent predicted $V_{A,eff}$. The relationship is significant (r = 0.491; P<0.001).



Appendix 4.6.3 Relationship between age and percent predicted $T_{L,CO,'sb'}$. The relationship is significant (r = 0.476; P<0.001).



Appendix 4.6.4 Relationship between age and percent predicted and $T_{L/VA}$. The relationship is not significant (r = 0.185; P>0.05).

Appendix 4.7 Results of curve fitting analysis applied to pressure-volume data from MVV-infected and control sheep. Data marked † is already represented in a previous table (Appendix 3.9) but is repeated here in the interests of clarity.

Sheep No.	r^2	Vmax	A	*K*
MVV08N	99.76	4717	3791	1.619
MVV002	96.08	2756	1861	0.711
MVV009	98.40	3330	1857	0.580
MVV029	98.86	3589	3021	1.631
MVV040	99.63	4667	2776	1.002
MVV047	98.94	2095	1479	0.332
MVV053	99.63	4249	2634	1.006
MVV065	98.92	3618	2838	1.412
MVV074	97.78	3156	2128	0.657
MVV075	94.16	3505	1936	1.527
MVV076	98.02	3461	2869	1.350
MVV080	98.28	3203	1774	1.626
CON089	99.10	4583	2981	1.386
CON090†	98.53	4204	2725	1.118
CON096	98.74	4150	2271	1.040
CON098	98.97	4902	3074	1.250
CON040	96.66	4716	2286	1.817
CON038†	98.86	4280	2597	1.087
CON091	99.80	4129	2443	1.581
CON099†	98.45	4104	1884	1.100
CON097†	99.21	4755	2403	0.984
CON088†	99.61	4177	2630	1.100
CON043†	98.97	3563	2138	2.005

Appendix 4.8 Results of Mann-Whitney U tests applied to the comparison of exponential coefficients from pressure-volume curve analysis of 11 control and 12 MVV-infected sheep.

Statistic	r^2	Vmax	A	*K*	A/Vmax
Mann-Whitney U Test Statistic	48.5	25.0	58.0	52.0	101.0
P	0.281	0.012	0.622	0.389	0.031

Appendix 5.1 Details of sheep used to provide material for macroscopic and microscopic morphometric evaluation of the lung. ND = not determined, the serological status of these ewes was unknown, however seronegativity is assumed given that these animals were purchased from an accredited MVV-free source.

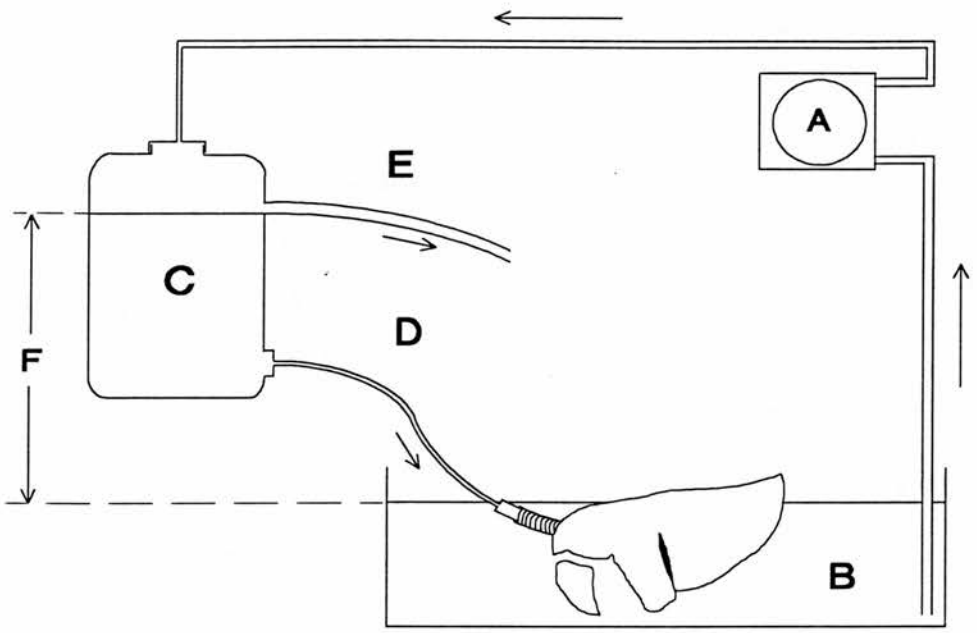
Sheep No.	Bodyweight	Breed	Sex	Seropositive for MVV?	Pathology No.
	(kg)			Y/N	
AHGR59	58	Suffolk cross	F	Y	BA 502/92
AHGR36	50	Mule	F	Y	BA 503/92
AHBL29	52	Welsh Mountain cross	F	Y	BA 504/92
AHBL28	69	Mule	F	Y	BA 508/92
AHBL97	53	Kerryhill	F	Y	BA 506/92
AHRD25	48	Kerryhill	F	Y	BA 509/92
MVV032	55	Texel	F	Y	BA 510/92
CON101	82	Oxford Texel cross	F	ND	BA 115/92
CON103	78	Oxford Texel cross	F	ND	BA 116/92
CON105	82	Oxford Texel cross	F	ND	BA 117/92

Appendix 5.2 Phosphate-buffered 4% paraformaldehyde was prepared using the following constituents.

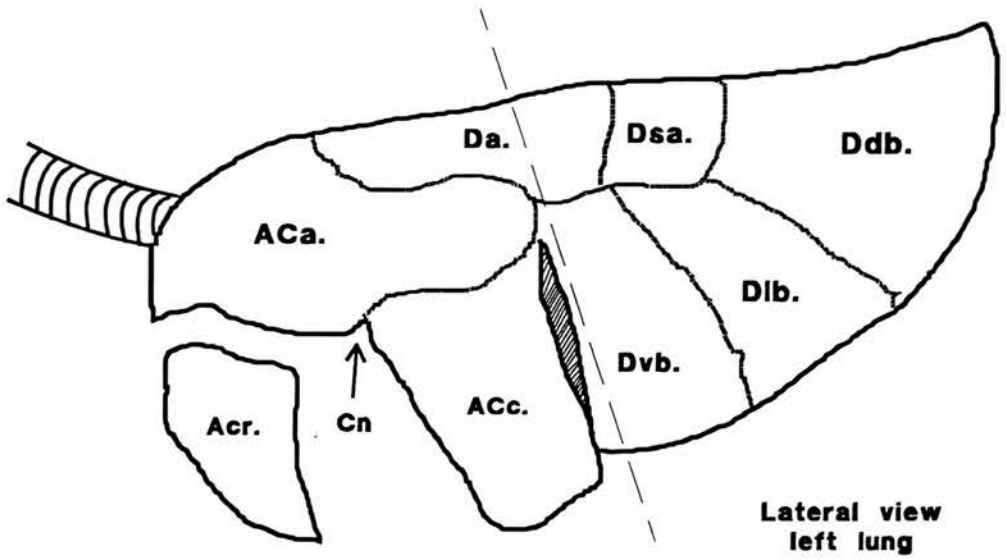
- Distilled water (to 10 litres)
- Paraformaldehyde (400g)
- Phosphate buffered saline (pH 7.2-7.4, x10 conc.) (1 litre)
- Sodium hydroxide (clearing agent)

Paraformaldehyde powder was gradually dissolved in hot distilled water and concentrated phosphate buffered saline. The solution was allowed to cool and then cleared using 1N sodium hydroxide and the pH adjusted to 7.2-7.4 using 1N sodium hydroxide and/or 1N hydrochloric acid. Between 20 and 40 litres of fixative were prepared according to the number of lungs to be fixed.

Appendix 5.3 Diagram of the apparatus used for inflation fixation of lungs at a constant hydrostatic pressure of 2.5-3.0 kPa for a period of 4 days. The peristaltic pump (A) circulates fixative from the large tank (B) containing the lungs to the height adjustable reservoir (C). The lungs are thus connected to a constant hydrostatic pressure of fixative by tubing (D). The pressure is adjusted by varying the height (F) of the reservoir (C) above the fixative level in the large tank (B). The pump (A) runs continuously and overflow runs off via a large bore pipe (E) back to the tank.

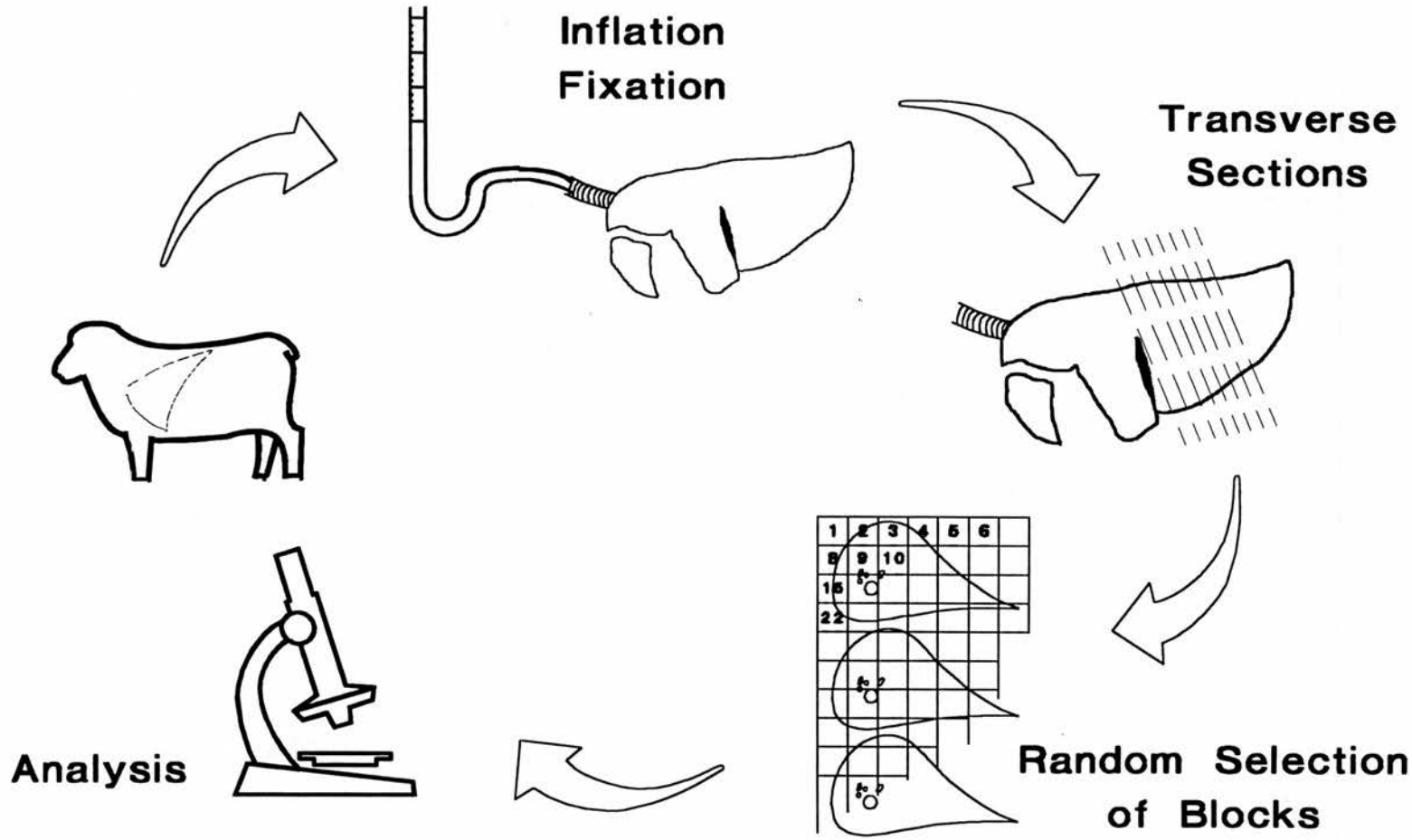


Appendix 5.4 Bronchopulmonary segments in the sheep lung according to Hare (1955). Six 1cm thick transverse lung slices were selected for tissue sampling from the region just caudal to the cranial margin of the ventral basal segment of the diaphragmatic lobe (Dvb.) i.e behind the dotted line.

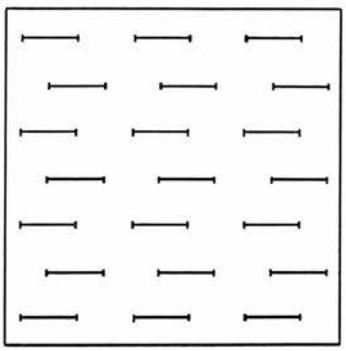


Acr.	Cranial segment of the right apical lobe
ACa.	Apical segment of the apico-cardiac lobe
ACc.	Cardiac segment of the apico-cardiac lobe
Dvb.	Ventral basal segment of the diaphragmatic lobe
Dlb.	Lateral basal segment of the diaphragmatic lobe
Ddb.	Dorsal basal segment of the diaphragmatic lobe
Da.	Apical segment of the diaphragmatic lobe
Dsa.	Subapical segment of the diphragmatic lobe

Appendix 5.5 (Overleaf) Diagrammatic summary of tissue fixation and sampling procedures. A transparent numbered grid overlay and computer generated random numbers were used to select 12 sites for sampling tissue blocks from the six slices.


Inflation
Fixation
Transverse
Sections
Random Selection
of Blocks
Analysis

Appendix 5.6 Method used in the morphometric analyses of histological sections. A multipurpose test grid (Weibel GW2: Graticules Ltd.) composed of a combined line and point system (Weibel *et al.*, 1966) was used in the quantification. The following method as described by Freere & Weibel (1966) was used to determine surface and volume densities. The multipurpose test grid (right) consists of a square frame enclosing 21 lines of equal length (Z) arranged in equidistant parallel rows. The total length of test line is L_T (21·Z). The 42 end points form a lattice for volumetric estimations while the lines provide probes for counting surface intersections per unit line.



Analyses were performed at a magnification of x400. Of the total number of end points counted (P_T) the number lying on tissue (P_t) or air (P_a) were recorded and the number of intersections of the test line with the tissue were also counted (N_t). A total of 72 randomly selected fields selected from a pool of 12 sections were examined in this way for each lung. Counts were totalled and used in the following calculations:

Volume density of air	V_{va}	P_a/P_T
Volume density of tissue	V_{vt}	P_t/P_T
Surface density of tissue	S_{vt}	$2 \cdot N_t/L_T$
Surface-to-volume ratio for tissue	s_t/v_t	$4 \cdot N_t/Z \cdot P_t$

The surface density of tissue is defined as the surface area of tissue per unit volume of lung and the surface-to-volume ratio is defined as the ratio of lung tissue surface area to lung tissue volume.

Appendix 5.7 Details of macroscopic point counting as applied to lung slices to estimate the volume of lung parenchyma (V_{vp}).

Sheep No.	No. of lung slices	Total no. of tissue hits	Total no. of parenchymal hits	V_{vp}	Total no. of non-parenchymal hits	Non-V_{vp}
				%		%
AHGR36	24	1583	1321	83.4	262	16.6
MVV032	18	1016	847	83.4	169	16.6
AHBL28	22	1200	1008	84.0	192	16.0
AHRD25	19	1063	882	83.0	181	17.0
AHBL97	20	992	873	88.0	119	12.0
AHGR59	23	1613	1369	84.9	244	15.1
AHBL29	18	1002	901	89.9	101	10.1

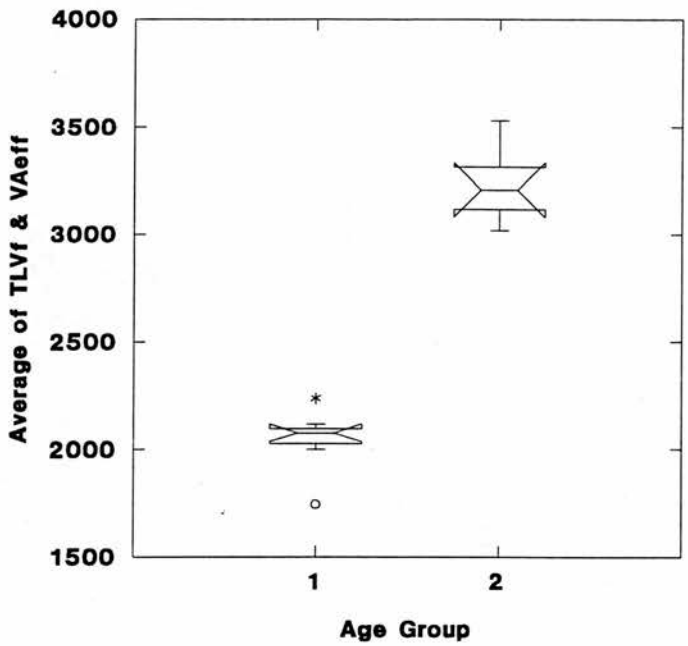
Appendix 5.8 Detailed results of microscopic point (P_t) and line intersection (N_t) counting executed on 3 normal sheep lungs. Six fields were randomly selected from each section for point counting and the totals shown for each section examined.

CON101			CON103			CON105		
Slide No.	P_t	N_t	Slide No.	P_t	N_t	Slide No.	P_t	N_t
8	59	173	10	32	176	5	54	192
5	42	142	11	52	177	11	63	246
1	38	164	9	34	190	7	60	194
10	44	151	7	35	185	11	59	261
5	47	171	8	62	140	2	59	224
2	30	161	11	38	128	3	69	175
8	51	151	2	39	177	6	49	177
7	60	160	7	56	269	8	69	155
2	44	200	9	47	189	11	64	226
3	38	196	3	52	148	5	62	177
1	68	185	9	47	173	7	72	193
5	54	160	4	46	194	9	49	215
Totals	575	2014	Totals	540	2146	Totals	729	2435

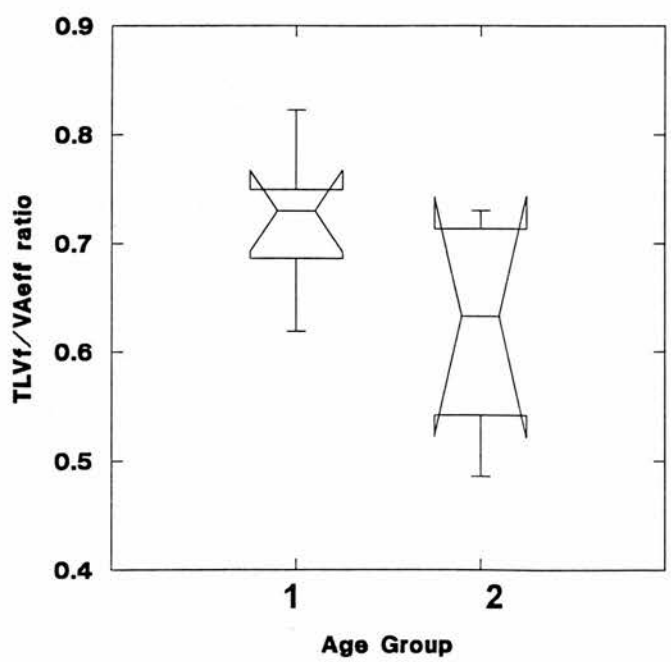
Appendix 6.1 Details of sheep used to determine the relationship between the time delay betwixt euthanasia and lung fixation and the ratio of fixed to physiological lung volume. AGED refers to sheep where the exact data of birth was not known but where the age was in excess of 5 years old. Sheep were grouped according to age, Group 1 less than or equal to 2 years old, Group 2 greater than or equal to 5 years old.

Sheep No	Breed	Sex	Age	Age Group	BW
-	-	-	-	-	kg
CON133	Texel Blackface cross	F	18 mo	1	46
CON136	Texel Blackface cross	F	18 mo	1	41
CON111	Finnish Landrace cross	F	2yrs	1	56
CON114	Finnish Landrace	MN	2yrs	1	60
CON138	Texel Blackface cross	MN	18 mo	1	40
CON132	Texel Blackface cross	F	18 mo	1	47
CON140	Texel Blackface cross	MN	18 mo	1	42
CON101	OxfordTexel cross	F	5yrs	2	82
CON103	OxfordTexel cross	F	5yrs	2	78
CON105	OxfordTexel cross	F	5yrs	2	82
CON145	Greyface	F	AGED	2	66
CON142	Greyface	F	AGED	2	67
CON144	Greyface	F	AGED	2	60

Appendix 6.2 Boxplot illustrating the dichotomy of the data set with respect to lung volume. there is a significant difference between the groups ($P<0.005$).



Appendix 6.3 Boxplot illustrating the non-independence of the two age groups with respect to the $TLV_F/V_{A,eff}$ ratio ($P = 0.063$) although a trend towards a lower ratio in the older age group is demonstrated.



Appendix 7.1 Clinical details of sheep used to examine pathophysiological correlations in maedi. The prefix ">" in the age column is used to indicate that the sheep were more than a given age at the time of the measurements, the actual birth date being unknown.

Sheep No.	BW		Age	Condition Score	Clinical Score
	(kg)		(yrs)		
MMV007	49		5.4	1.0	0
MVV012	67	>	6.9	1.5	0
MVV017	50		5.2	1.5	1
MVV032	55		6.7	2.0	0
MVV053	50		5.9	2.0	2
MVV059	52	>	7.3	1.0	1
MVV068	52		6.0	1.0	0
MVV073	50		6.5	1.0	0
MVV074	60		6.0	1.0	2
MVV079	51		5.4	1.5	2
MVV089	65	>	6.9	2.5	1
MVV090	67		5.9	3.0	0
MVV092	64		5.9	2.0	0
MVV093	62	>	6.9	2.5	0
MVV096	62		5.9	2.5	1
MVV097	60		5.9	2.5	0

Appendix 7.2 (Overleaf) Morphometric data derived from the study of 16 lungs from sheep seropositive for MVV. The symbols are those represented in chapter 7, figures 7.1-7.5.

Appendix 7.3 (Turn 2 pages) Physiologic data derived from functional measurements made in 16 sheep seropositive for MVV. Values in parenthesis are % predicted values.

Appendix 7.2

Sheep No.	Symbol	Total Lung Weight	Total Lung Volume	Volume of lung parenchyma	Tissue volume fraction	V_{vt}:V_{v} a ratio	Total parenchymal airspace volume	Surface density	Surface to volume ratio for tissue	Total airspace surface area of lung parenchyma
		TLW ml	TLV_F ml	TV_p ml	V_{vt} %	-	$TV_{p,a}$ ml	S_{vt} cm^2/cm^3	s_t/v_t cm^2/cm^3	ASA_p m^2
MVV007	○	0.81	2498	2123	26.0	0.35	1572	537.6	2071.1	114.1
MVV012	◍	0.72	2188	1859	22.0	0.28	1451	684.9	3119.1	127.4
MVV017	⊖	0.86	3194	2715	23.5	0.31	2077	542.9	2309.1	147.4
MVV032	⊕	0.55	1750	1488	18.0	0.22	1451	596.4	3309.3	105.5
MVV053	⊘	1.32	2844	2417	31.9	0.47	1645	410.1	1283.6	99.1
MVV059	⊘	0.84	2716	2309	24.0	0.32	1754	534.1	2221.6	123.3
MVV068	⊙	1.35	2651	2253	43.4	0.77	1276	374.2	863.1	84.3
MVV073	◑	1.20	3646	3099	29.1	0.41	2198	505.6	1739.4	156.7
MVV074	◐	1.74	3055	2597	50.2	1.01	1294	265.4	529.1	68.9
MVV079	◓	1.39	2352	1999	48.7	0.95	1025	243.4	499.7	48.7
MVV089	◒	0.60	2370	2014	17.8	0.22	1655	578.5	3245.5	116.5
MVV090	●	0.64	2643	2247	19.7	0.25	1803	676.4	3426.0	152.0
MVV092	◔	0.62	2625	2231	17.0	0.21	1851	555.8	3263.9	124.0
MVV093	◔	0.60	1823	1550	24.9	0.33	1164	671.4	2699.8	104.0
MVV096	✚	0.96	2534	2154	23.5	0.31	1648	673.1	2867.0	145.0
MVV097	✳	0.61	2425	2061	17.5	0.21	1700	519.7	2965.2	107.1

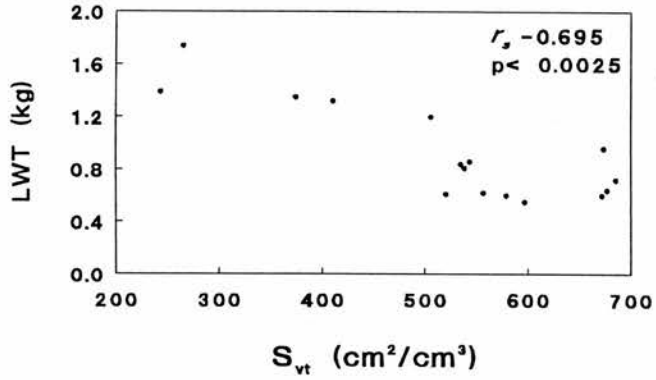
Appendix 7.3

Sheep No.	Static lung compliance		Transfer factor for carbon monoxide		Transfer factor per litre of effective alveolar volume		Effective alveolar volume		Index of lung distensibility	Index of lung recoil
	Cst		$T_{L,CO,'sb'}$		$T_{L/VA}$		$V_{A,eff}$		*K*	A/Vmax
	l/kPa	% Predicted	mmol/min/kPa	% Predicted	mmol/min/kPa/l	% Predicted	l	% Predicted	-	-
MVV007	1.452	(100)	6.42	(74)	1.78	(80)	3.61	(91)	1.343	0.730
MVV012	2.263	(125)	7.39	(69)	1.59	(64)	4.64	(108)	1.447	0.601
MVV017	1.554	(106)	9.51	(108)	2.06	(92)	4.62	(116)	1.441	0.661
MVV032	1.671	(107)	8.09	(87)	2.12	(92)	3.82	(93)	1.524	0.691
MVV053	0.887	(61)	2.61	(30)	0.88	(39)	2.97	(74)	1.460	0.774
MVV059	1.327	(88)	5.53	(62)	1.57	(69)	3.52	(87)	1.307	0.541
MVV068	1.026	(68)	3.12	(35)	1.09	(48)	2.85	(71)	0.860	0.696
MVV073	1.116	(76)	7.01	(80)	1.67	(75)	4.19	(105)	1.303	0.722
MVV074	1.166	(70)	2.96	(30)	1.10	(46)	2.69	(64)	0.912	0.659
MVV079	0.690	(46)	2.07	(23)	1.10	(49)	1.88	(47)	0.905	0.532
MVV089	0.672	(38)	6.67	(64)	1.96	(80)	3.40	(80)	0.685	0.564
MVV090	1.581	(87)	9.13	(86)	1.91	(77)	4.77	(111)	1.866	0.541
MVV092	2.705	(155)	7.71	(75)	1.74	(71)	4.43	(104)	1.794	0.603
MVV093	2.189	(128)	6.89	(68)	1.66	(69)	4.15	(98)	1.656	0.455
MVV096	1.482	(87)	7.49	(74)	1.94	(81)	3.86	(92)	1.119	0.420
MVV097	2.406	(144)	8.78	(89)	1.92	(81)	4.58	(110)	1.843	0.467

Appendix 7.4 Spearman rank-order correlation coefficients (r_s) and levels of significance of the relationships between clinical, and morphometric and physiological data.

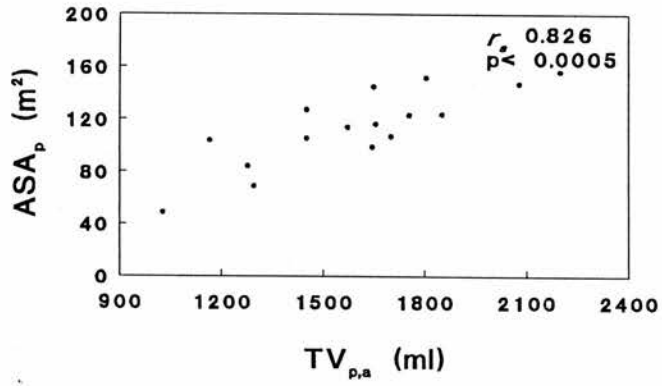
Variable	Body condition score		Clinical score		Bodyweight	
	r_s	P	r_s	P	r_s	P
TLW	-0.557	P<0.025	0.615	P<0.01	-0.475	NS
TLV_F	-0.409	NS	0.375	NS	-0.424	NS
V_{vt}	-0.590	P<0.01	0.509	P<0.025	-0.575	NS
TV_{pa}	0.128	NS	-0.040	NS	-0.006	NS
S_{vt}	0.601	P<0.01	-0.312	NS	0.662	P<0.005
s_t/v_t	0.686	P<0.0025	-0.522	P<0.025	0.683	P<0.0025
ASA_p	0.004	NS	-0.216	NS	0.216	NS
Cst	0.366	NS	-0.462	P<0.05	0.434	NS
V_{Aeff}	0.429	NS	-0.446	P<0.025	0.397	NS
$T_{LCO'sb'}$	0.510	P<0.025	-0.379	NS	0.359	NS
$T_{L/VA}$	0.499	P<0.025	-0.168	NS	0.547	NS
K	0.496	P<0.05	-0.502	P<0.05	0.254	NS
A/Vmax	-0.573	P<0.025	-0.055	NS	-0.591	P<0.01

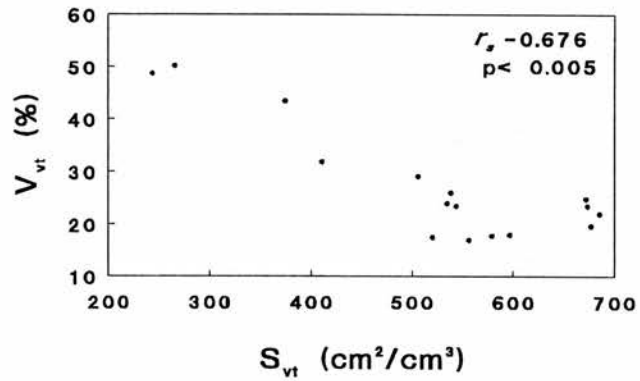
Appendix 7.5.1 - 7.5.4 (overleaf) Correlations between total lung weight (TLW) and tissue surface density (S_{vt})(r_s -0.695; P<0.0025)(Appendix 7.5.1), total parenchymal airspace volume ($TV_{p,a}$) and total airspace surface area of lung parenchyma (ASA_p)(r_s 0.826; P<0.0005)(Appendix 7.5.2), S_{vt} and tissue volume fraction (V_{vt})(r_s -0.676; P<0.005)(Appendix 7.5.3) and S_{vt} and ASA_p (r_s 0.591; P<0.01)(Appendix 7.5.4).



Appendix 7.5.1 (left)

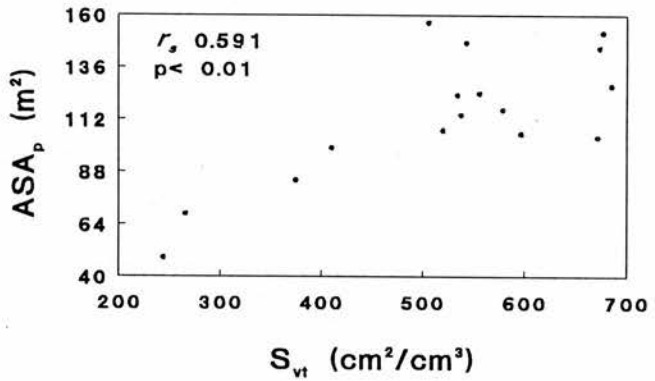
Appendix 7.5.2 (right)





Appendix 7.5.3 (left)

Appendix 7.5.4 (right)

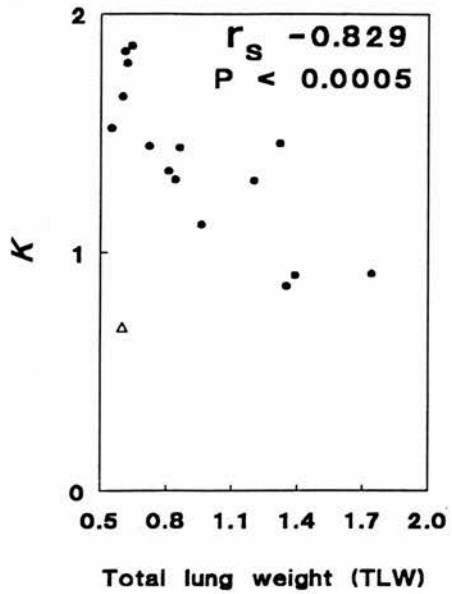


Appendix 7.6 Spearman rank-order correlation coefficients (r_s) and levels of significance of the relationships between morphometric variables.

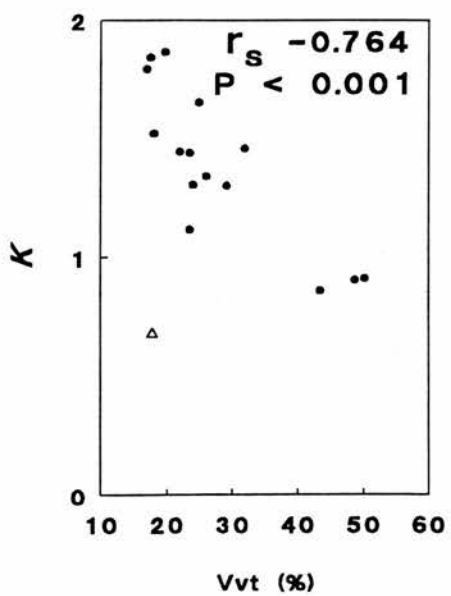
	TLW	TLV_F	V_{vt}	$TV_{p,a}$	S_{vt}	ASA_p
TLW	—	0.629 P<0.01	0.829 P<0.0005	-0.167 NS	-0.693 P<0.0025	-0.258 NS
TLV_F		—	0.388 NS	0.547 NS	-0.456 NS	0.279 NS
V_{vt}			—	-0.482 NS	-0.676 P<0.005	-0.497 NS
$TV_{p,a}$				—	0.200 NS	0.826 P<0.0005
S_{vt}					—	0.591 P<0.01
ASA_p						—

Appendix 7.7 Spearman rank-order correlation coefficients (r_s) and levels of significance of the relationships between physiological variables.

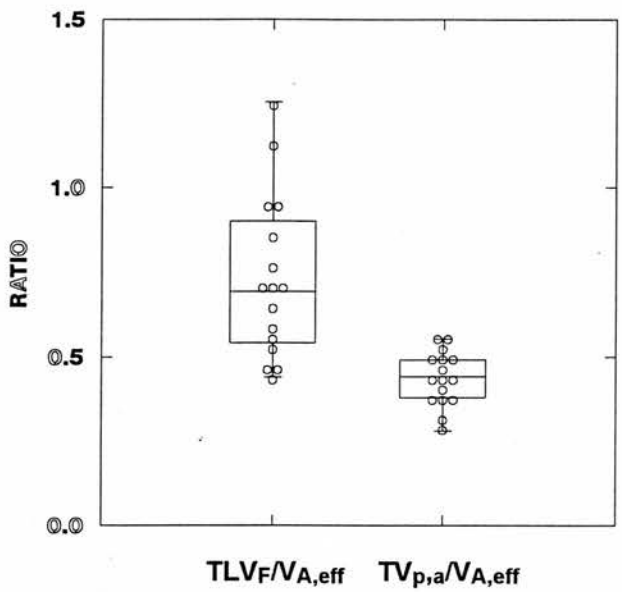
	Cst	K	A/Vmax	$T_{L,CO,'sb'}$	$T_{L/VA}$	$V_{A,eff}$
Cst	—	0.800 P<0.0005	-0.300 NS	0.726 P<0.0025	0.379 NS	0.765 P<0.001
K		—	-0.116 NS	0.618 P<0.01	0.250 NS	0.706 P<0.0025
A/Vmax			—	-0.218 NS	-0.209 NS	-0.200 NS
$T_{L,CO,'sb'}$				—	0.794 P<0.0005	0.882 P<0.005
$T_{L/VA}$					—	0.521 NS
$V_{A,eff}$						—



Appendix 7.8 & 7.9 Demonstrating the relationships between K and total lung weight (TLW)(above), and K and tissue volume fraction (V_{vt})(below) respectively. Sheep No. 89 (open triangle) is excluded from the data set in the computation of the Spearman-rank correlation coefficients (see discussion).



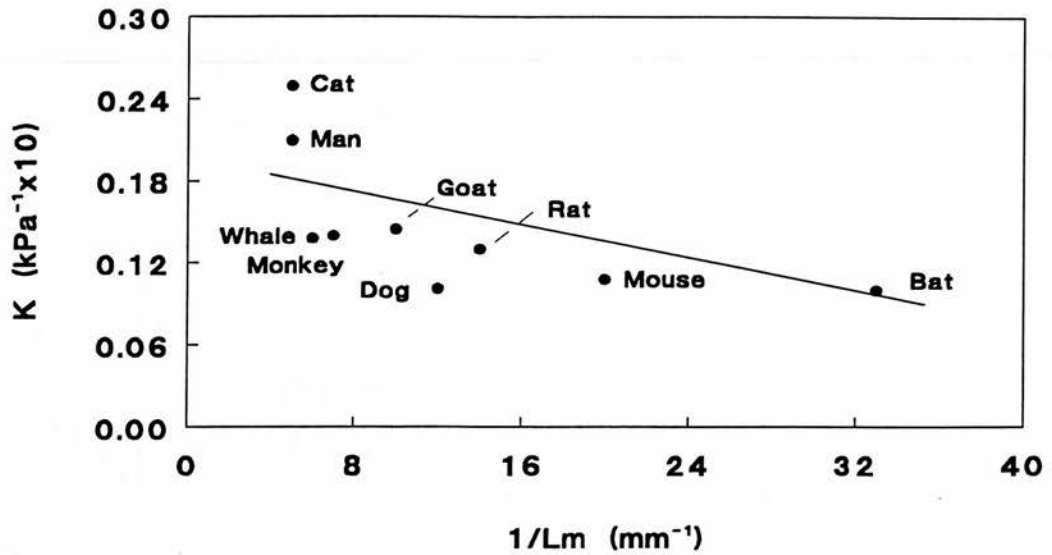
Appendix 7.10 Boxplots illustrating the range of values for the fixed to physiological lung volume ratio ($TLV_F/V_{A,eff}$)(median 0.692; range 0.440-1.253) and the ratio of total parenchymal airspace volume to physiological lung volume ($TV_{p,a}/V_{A,eff}$)(median 0.442; range 0.281-0.553).)



Appendix 7.11 Haber *et al.* (1983) related measurements of K made in excised lungs to the mean linear intercept (L_m). This latter parameter is a morphometric estimate of the mean size of peripheral airspaces at maximal inflation. It is obtained by dividing the total length of test line placed on the surface under study by the number of intersections the test line makes with the surface component of interest i.e. it is the mean distance between intersections and this measurement can be shown to be inversely proportional to the total surface area of the component making the intersections (Aherne & Dunnill, 1982). The surface area of a component per unit volume (surface density (S_v)) is given by

$$2/L_m = 2N/L$$

where N is the number of intersections with a test line of length L. Haber *et al.* (1983) showed that L_m correlated highly with K in air-filled lungs but could demonstrate no relationship in saline-filled lungs. These authors concluded that tissue elastic properties did not normally determine lung distensibility and that the density of surface forces was more relevant than that of tissue elements in determining lung recoil. Appendix 7.1.1 is adapted from figure 5 in the study by Haber *et al.* (1983). Data points were measured from the respective axes and the reciprocal values of L_m calculated and plotted against K.



Appendix 7.11.1 Adapted from Haber *et al.* (1983). This graph demonstrates that species with small alveoli (e.g. bats) tend to have low values for lung distensibility in contrast to species with large alveoli (e.g. man).

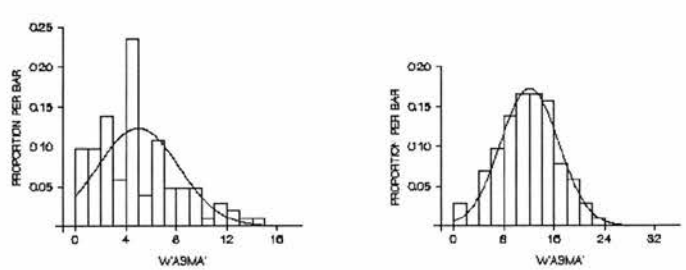
Appendix 8.1.1 (overleaf) Summary statistics from microscopic point counting using a 121 point grid to estimate the tissue volume fraction (V_{vt}) in lung parenchyma. Compounded standard errors were calculated according to the formula

$$\text{Compounded S.E.} = 1/N \left[\sum_i N_i P_i (100 - P_i) \right]^{1/2}$$

where N is the total number of points counted, P_i is the percentage of points falling on a given component in the i^{th} field, and N_i is the total number of points applied to that field (Anderson & Dunnill, 1965; Aherne & Dunnill, 1982). The relative S.E. is the compounded standard error divided by the mean. A positive measure of skewness (G1) indicates skewness to the right. The significance of the sample values of t (t_S) for measures of skewness are given in superscript (a - $P<0.001$, b - $P<0.01$, c - $P<0.05$, NS - $P>0.05$)(Sokal & Rohlf, 1981). The coefficent of variation (C.V.) is the standard deviation divided by the mean. All statistical parameters were calculated using commercially available statistical software (Systat for Windows, v.5; Systat Inc., Evanston, IL, USA).

Appendix 8.1.2 (two pages overleaf) Summary statistics from microscopic point counting using a 121 point grid to estimate the ASMA volume fraction ($V_{v'ASMA'}$) in lung parenchyma. Statistics were calculated as for appendix 8.1.1.

Appendix 8.1.3 The Lilliefors adaption of the Kolmogorov-Smirnov test was used as a test for normal distribution of variables. Strictly speaking, the Kolmogorov-Smirnov test assumes a continuously distributed variable, however as pointed out by Sokal & Rohlf (1981) it is often used for discrete variables. Given the fact that grid points landing on ASMA expressing tissue were rare events it was expected that $V_{v'ASMA'}$ values would follow a Poisson distribution. Attempted arcsine transformations failed to correct these distributions to normal in all but one instance (below) therefore nonparametric statistical tests were used in further data analysis.



Arcsine transformation of $V_{v'ASMA'}$ values for MVV068 corrected the initial right skewed data (left histogram) to a normal distribution (right histogram) (n=102, maximum difference = 0.078, Lilliefors 2-tailed probability = 0.122).

Appendix 8.1.1

	MVV007	MVV012	MVV017	MVV032	MVV053	MVV059	MVV068	MVV073	MVV074
No of fields	100	104	132	232	100	132	102	100	100
Minimum	11.57	15.702	9.917	6.612	13.223	10.744	14.05	14.876	18.182
Maximum	69.421	38.017	61.983	65.289	85.95	87.603	83.471	88.43	100
Mean	27.843	25.079	26.039	19.842	44.529	27.767	41.144	31.81	46.901
Variance	97.232	27.198	56.471	65.191	352.246	77.132	232.111	121.142	354.993
Standard deviation	9.861	5.215	7.515	8.074	18.768	8.782	15.235	11.006	18.841
Standard error	0.986	0.511	0.654	0.53	1.877	0.764	1.509	1.101	1.884
Compound S.E.	0.048	0.045	0.040	0.027	0.061	0.042	0.058	0.051	0.062
Relative S.E.	0.172	0.178	0.155	0.134	0.136	0.150	0.140	0.161	0.132
Skewness (G1)	1.634	0.285	1.761	2.211	0.391	2.665	1.167	2.665	1.25
*t*s	6.77[a]	1.20[NS]	8.35[a]	13.84[a]	1.62[NS]	12.64[a]	4.88[a]	11.04[a]	5.18[a]
C.V.	0.354	0.208	0.289	0.407	0.421	0.316	0.37	0.346	0.402
Median	26.446	24.793	24.793	19.008	40.496	26.033	37.19	29.752	42.975

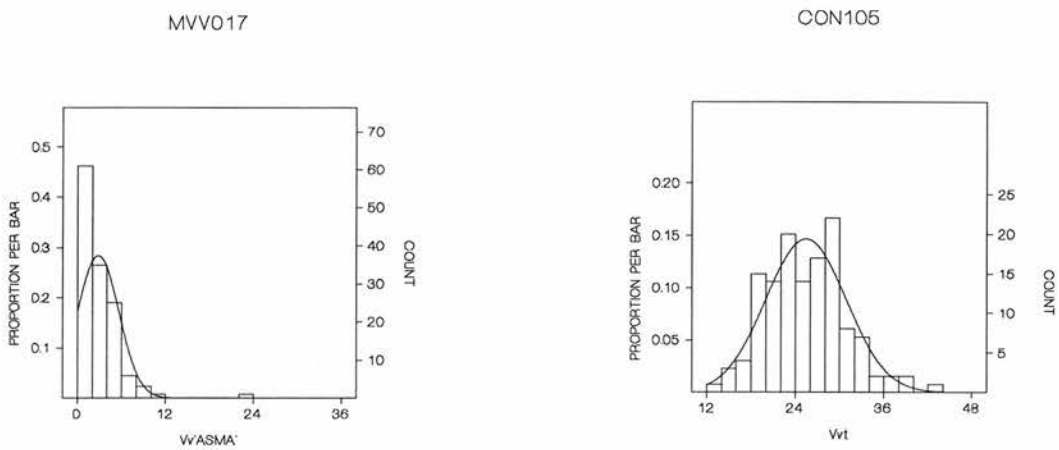
	MVV079	MVV089	MVV090	MVV092	MVV096	MVV097	CON101	CON103	CON105
No of fields	100	132	181	132	100	140	208	241	132
Minimum	18.182	11.57	10.744	8.264	15.702	8.264	11.57	8.264	12.397
Maximum	100	55.372	45.455	83.471	67.769	33.884	41.322	52.893	42.149
Mean	47.289	24.149	24.661	20.173	26.215	19.268	22.39	23.881	25.457
Variance	248.832	30.463	39.259	59.312	57.291	29.906	24.705	30.908	29.546
Standard deviation	15.774	5.519	6.266	7.701	7.569	5.469	4.97	5.559	5.436
Standard error (S.E.)	1.577	0.48	0.466	0.67	0.757	0.462	0.345	0.358	0.473
Compound S.E.	0.062	0.039	0.034	0.035	0.046	0.034	0.030	0.029	0.040
Relative S.E.	0.132	0.161	0.136	0.176	0.177	0.175	0.133	0.120	0.157
Skewness (G1)	1.07	1.284	0.771	4.301	1.963	0.442	0.578	1.064	0.324
*t*s	4.43[a]	6.09[a]	4.27[a]	20.40[a]	8.13[a]	2.16[c]	3.43[a]	6.79[a]	1.54[NS]
C.V.	0.334	0.229	0.254	0.382	0.289	0.284	0.222	0.233	0.214
Median	45.455	23.967	23.967	19.835	25.62	19.008	21.488	23.14	25.62

Appendix 8.1.2

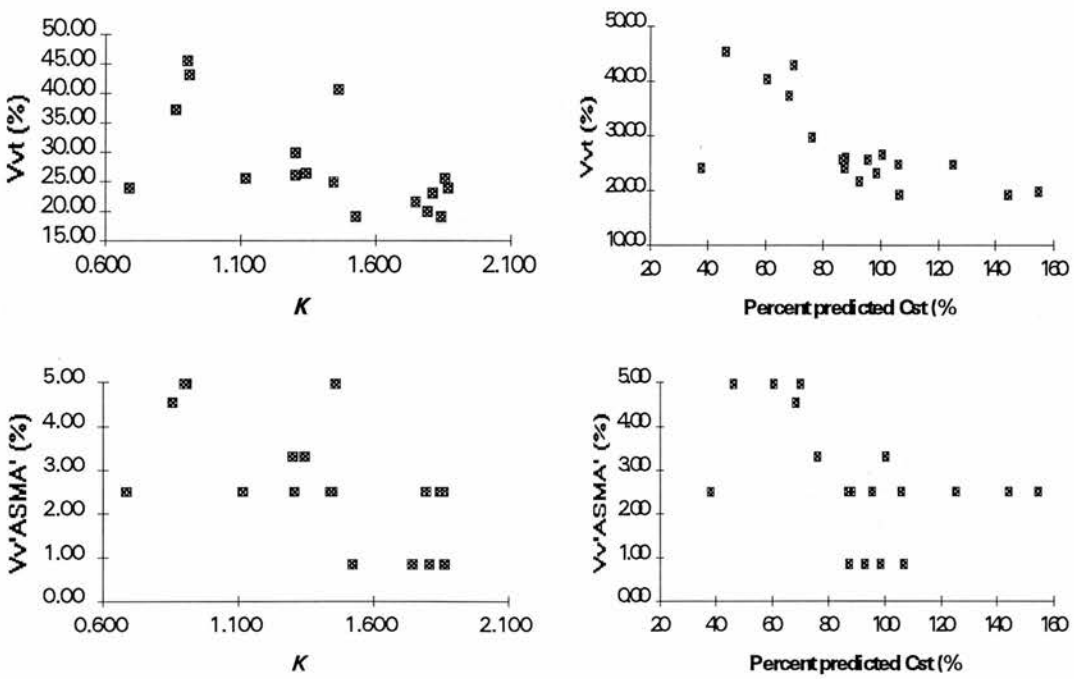
	MVV007	MVV012	MVV017	MVV032	MVV053	MVV059	MVV068	MVV073	MVV074
No of fields	100	104	132	232	100	132	102	100	100
Minimum	0	0	0	0	0	0	0	0	0
Maximum	15.702	8.264	22.314	11.57	17.355	10.744	14.05	10.744	25.62
Mean	3.744	3.163	2.83	1.621	5.24	2.467	4.959	3.678	5.545
Variance	6.05	4.913	7.885	4.461	13.939	4.108	10.468	6.697	16.783
Standard deviation	2.46	2.217	2.808	2.112	3.734	2.027	3.235	2.588	4.097
Standard error	0.246	0.217	0.244	0.139	0.373	0.176	0.32	0.259	0.41
Compound S.E.	0.018	0.016	0.013	0.008	0.021	0.012	0.02	0.017	0.021
Relative S.E.	0.47	0.501	0.47	0.469	0.397	0.504	0.404	0.474	0.386
Skewness (G1)	1.493	0.674	3.034	1.821	0.659	1.18	0.733	0.832	1.423
t_S	3.77	1.76	7.68	5.54	1.53	3.30	1.74	2.07	3.27
C.V.	0.657	0.701	0.992	1.303	0.713	0.822	0.652	0.704	0.739
Median	3.306	2.479	2.479	0.826	4.959	2.479	4.545	3.306	4.959

	MVV079	MVV089	MVV090	MVV092	MVV096	MVV097	CON101	CON103	CON105
No of fields	100	132	181	132	100	140	208	241	132
Minimum	0	0	0	0	0	0	0	0	0
Maximum	14.876	9.091	9.091	19.008	14.876	8.264	9.091	10.744	6.612
Mean	5.446	2.692	1.849	2.661	3.38	2.397	1.367	1.382	2.623
Variance	12.296	3.99	4.329	6.656	7.328	3.767	2.439	3.071	2.633
Standard deviation	3.507	1.997	2.081	2.58	2.707	1.941	1.562	1.752	1.623
Standard error	0.351	0.174	0.155	0.225	0.271	0.164	0.108	0.113	0.141
Compound S.E.	0.021	0.013	0.009	0.013	0.017	0.012	0.007	0.007	0.013
Relative S.E.	0.389	0.482	0.497	0.485	0.494	0.496	0.539	0.498	0.488
Skewness (G1)	0.56	0.992	1.419	2.88	1.526	0.753	1.582	1.926	0.387
t_S	1.31	2.79	4.15	7.46	3.76	2.16	5.31	6.35	1.17
C.V.	0.644	0.742	1.125	0.97	0.801	0.81	1.143	1.268	0.619
Median	4.959	2.479	0.826	2.479	2.479	2.479	0.826	0.826	2.479

Appendix 8.2 Distributions of $V_{v'ASMA'}$ values typically approximated to the Poisson distribution whereas V_{vt} values more often approximated to the normal distribution. Typical examples of each type of distribution are shown below.



Appendix 8.3 Relationships between V_{vt} and K and between V_{vt} and Cst (expressed as percent predicted) (r_s = -0.615; p<0.005 and r_s = -0.683; P<0.0025 respectively (one-tailed)). Also shown are the relationships between $V_{v'ASMA'}$ and K and between $V_{v'ASMA'}$ and Cst (% predicted)(r_s = -0.614; p<0.005 and r_s = -0.504; P<0.025 respectively (one-tailed)).



Appendix 9.1 Details of sheep used to study the relationship between the quantity and functional tone of parenchymal contractile tissue and lung elastic properties in sheep seronegative for MVV and sheep seropositive for MVV. The last three columns indicate which experimental protocols (Chapter 9; section 9.2.6) the sheep were utilized for together with which sheep were subsequently euthanazed to allow morphometric examination of the lungs.

Sheep No.	BW (kg)	Breed	Year of birth	Serological Status		Hist. admin.	Clen. admin	P.M.
				Result	Test Date			
CON089	80	TEXEL	1986	-ve	20/4/93	✓		
CON091	70	TEXEL	1984	-ve	20/4/93	✓		
CON092	93	TEXEL	1985	-ve	20/4/93	✓		
CON094	65	TEXEL	1989	-ve	20/4/93	✓		
CON097	57	TEXEL	1984	-ve	20/4/93	✓	✓	✓
CON098	87	TEXEL	1984	-ve	20/4/93		✓	✓
MVV004	49	TEXEL	1989	+ve	30/11/93	✓		✓
MVV021	73	TEXEL	1987	+ve	20/4/93	✓	✓	✓
MVV026	81	TEXEL	1987	+ve	20/4/93	✓	✓	✓
MVV039	71	TEXEL	1986	+ve	20/4/93	✓	✓	✓
MVV040	72	TEXEL	1986	+ve	20/4/93	✓	✓	
MVV058	68	TEXEL	1986	+ve	20/4/93	✓	✓	✓
MVV100	63	TEXEL	1986	+ve	20/4/93	✓	✓	✓
MVV130	63	TEXEL	1986	+ve	20/4/93	✓	✓	✓

Appendix 9.2 LabView is a software system that uses a graphic interface to allow users to build software modules called virtual instruments (VIs) which can control data acquisition and execute data analysis and presentation. No text-based programming ability is required as programmes are drawn in graphical block diagrams which are easy to understand, modify and maintain.

The principal VI ('DDSC POLY5') that was constructed to facilitate real-time data acquisition and analysis in this study was an adaptation of a VI used to monitor cardiovascular status in anaesthetized horses. This VI was developed by Dr Hamish Ross (Birmingham, UK) for the Department of Veterinary Clinical Studies, R.(D).S.V.S. (see acknowledgements). Analysis and display routines within this VI that were maintained concerned the mean blood pressure and the heart rate trace. Aside from minor modifications to board specific VIs, the development of a respiratory function analysis subVI was vital to the success of this real-time analysis VI. It is this subVI, 'R_L and Cdyn CALC', that will be discussed in more detail.

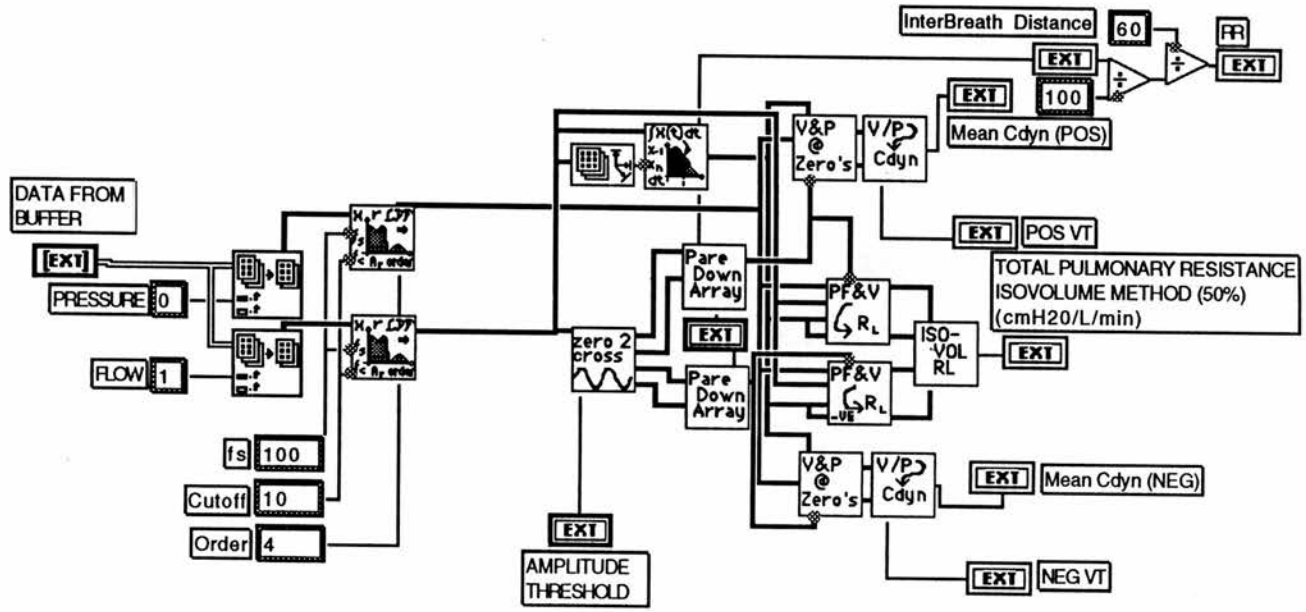
DDSC POLY5

Overview: When this VI is started the first action that is undertaken is to configure the ADC board to undertake double-buffered data acquisition and to allocate a buffer size within memory for continuous acquisition of samples (DAQ2Config). Errors detected at any stage will result in DAQ2Clear operating, this VI effectively reversing all the changes previously instituted. An interactive dialog is then displayed which prompts the operator to open a new folder for results and writes appropriate headers in this folder for summary analysis results. The next sequence institutes a multiple channel data acquisition operation in double-buffered mode (Lab_SCANStart). At this stage the number of channels, the gain settings and the sampling rate are specified. The A/D conversions are acquired and stored within the large buffer configured earlier. This data acquisition wil run continually until the stop button on the front panel is pressed. A sampling, display and analysis routine then runs continuously in which a VI (DAQ2TGet) returns a specified block of data from the buffer at 20 second intervals. It is this data that is submitted to various subVIs for analysis. When the record button on the front panel is pressed a subVI runs which displays a given time period (determined by the duration control) of raw data on the monitor screen. The results of analysis of this raw data are stored within the file specified in the earlier interactive sequence. Summary analysis results are only stored to file when the record button is pressed i.e. the data displayed on screen is not stored. Every 20 seconds, the VI 'R_L and Cdyn CALC executes and returns data to the front panel for graphic display or stores the data if the record button is pressed.

R_L and Cdyn CALC

Overview: This VI examines the pressure and flow arrays acquired as blocks from the circular buffer and identifies inspiratory and expiratory breaths on the basis of flow zero crossing points and interactively defined thresholds for breath duration and crossing point limits. Integration of the flow trace between zero crossing points yields respiratory volumes and calculation of R_L, and C_{dyn} is based on accepted methods (Appendix 9.3). Both pressure and flow arrays were filtered at 10Hz using an elliptic low pass filter prior to analysis. The block diagram of R_L and Cdyn CAL is shown overleaf and descriptions of the individual component subVIs follow.

Zero 2 cross VI: This VI identifies and categorizes zero crossing points into positive to negative and negative to positive zero crossings. A zero crossing is identified if two consecutive values in the array that proceed or preceed a crossing of an interactively set threshold land either side of zero.



Appendix 9.2 contd. R_L and Cdyn CALC VI block diagram. This VI calculates dynamic compliance (C_{dyn}), total pulmonary resistance (R_L), tidal volume (V_T) and respiratory rate (RR) from blocks of data returned from the circular buffer. Calculated values are logged to disk and/or displayed on the monitor strip charts and digital indicators.

Appendix 9.2 contd. (Zero 2 crossing VI)

These identified zero crossing points are the first stage in delimiting the breath.

Pare Down Array VI: This VI applies a further constraint to the acceptance of zero crossing points, namely that they have to be separated by a given time interval. This eliminates spurious zero crossings that arise as a result of underdamped flow recordings i.e. 'spikes'. When the duration threshold is set to about 0.5 secs, only crossings at either 'side' of normal breaths are accepted and returned by this VI. Further tasks executed by this VI are to slice off partly recorded breaths at the start or end of the recording period and to calculate the interbreath distance i.e. the average time period between consecutive breaths commencing.

V & P @ Zero's VI: This VI uses the indices of zero crossing points returned by the Pare Down Array VI to assemble arrays of pressures and volumes at these points.

V/P $\rightarrow$ Cdyn VI: Divides the volume change between points of zero flow by the corresponding pressure change and calculates the mean value for the breaths examined to give the dynamic compliance in units of volume per unit pressure. The mean volume change between zero crossings is also returned as the tidal volume. A simple calculation based on the sampling rate allows calculation of the respiratory rate.

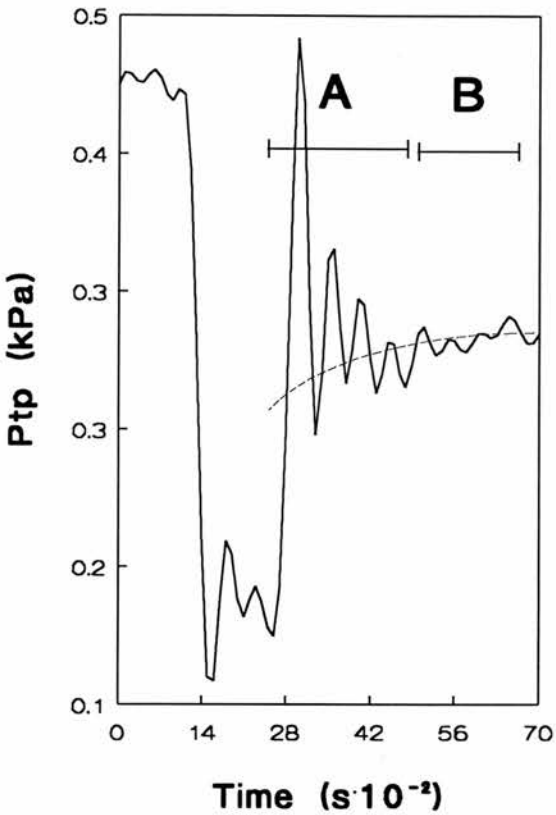
PF&V $\rightarrow$ R_L VI: Identifies and assembles into arrays the pressure and flow values at mid-tidal volume points (IsoVol 50%).

ISO-VOL R_L VI: Calculates the absolute difference between pressure and flow values at the IsoVol 50% points and divides the former by the latter to give the total pulmonary resistance in units of pressure per unit flow.

Appendix 9.3 References for the methods used by the R_L and Cdyn CALC VI in calculating dynamic compliance and total pulmonary resistance.

Computed parameter	Abbreviation	Definition
Dynamic compliance	C_{dyn}	The volume change per unit pressure change between points of zero flow (Krieger, 1963)
Total pulmonary resistance	R_L	The ratio of pressure change to corresponding flow change between isovolume points (Frank *et al.*, 1957).

Appendix 9.4 It is apparent that the transient response curve of Pao on rapid interruption of air flow is characterized by two regions (opposite). The first region (A) is characterized by an initial high frequency oscillation and is followed by a slow change in Pao (B). According to Jackson *et al.* (1974) the first component is due to the oscillation of the airways and the second is due to tissue dynamics. By performing a backwards curvilinear extrapolation of the mean pressure during the oscillatory phase (dotted line), it is possible to estimate Pao at the time of interruption and this value serves as an estimate of alveolar pressure. In accepting this method as providing a true estimate of alveolar pressure, the corresponding conclusion regarding the ratio of alveolar pressure to respiratory flow at the point of interruption is that this ratio is a true reflection of airways resistance.



Appendix 9.5 (overleaf) Results from off-line analysis of data collected just before (PRE) and immediately after (POST) a 5 minute histamine infusion sufficient to reduce dynamic lung compliance to 65% of baseline values.

Appendix 9.6 (two pages overleaf) Arterial blood gas, acid-base, blood pressure, heart rate and total pulmonary resistance values obtained immediately before (PRE) and immediately after (POST) a 5 minute histamine infusion sufficient to reduce dynamic lung compliance to 65% of baseline values or increase total pulmonary resistance twofold.

Sheep ID	Time Point	K	A/Vmax	E_{oes}	$V_{A,eff}$	$T_{L,CO,'sb'}$	$T_{L/VA}$	R_{int}	Cst
				kPa/ml	l	mmol/min/kPa	mmol/min/kPa/l	kPa/l/s	ml/kPa
MVV040	PRE	1.256	0.443	0.079	4.258	25.30	5.94	1.131	2100
MVV040	POST	1.145	0.466	0.085	4.087	25.81	6.31	6.761	1860
CON089	PRE	1.664	0.529	0.085	3.588	19.22	5.36	1.288	2900
CON089	POST	2.099	0.573	0.089	3.713	23.07	6.21	1.504	3430
CON091	PRE	1.797	0.596	0.158	4.189	33.56	8.01	1.122	3600
CON091	POST	1.505	0.537	0.268	4.115	33.38	8.11	0.895	4800
CON092	PRE	0.498	1.032	0.051	1.891	13.45	7.11	1.989	710
CON092	POST	0.348	0.917	0.046	1.177	9.95	8.45	1.901	410
CON094	PRE	1.912	0.6	0.105	3.348	24.18	7.22	0.978	3080
CON094	POST	1.160	0.658	0.079	2.679	18.69	6.98	2.416	1500
CON097	PRE	1.427	0.496	0.119	4.186	27.21	6.50	2.063	5800
CON097	POST	1.444	0.496	0.079	4.136	23.98	5.80	1.559	3400
MVV004	PRE	1.556	0.611	0.381	3.485	24.76	7.10	0.515	3750
MVV004	POST	1.570	0.641	0.120	3.204	27.07	8.45	0.726	2500
MVV021	PRE	1.149	0.395	0.470	4.122	28.70	6.96	1.090	2040
MVV021	POST	1.018	0.467	0.421	2.505	37.12	14.82	1.097	1400
MVV026	PRE	1.807	0.52	0.106	4.315	29.54	6.85	1.365	3000
MVV026	POST	1.347	0.452	0.087	4.195	36.62	8.73	1.551	1700
MVV039	PRE	1.554	0.501	0.136	5.280	39.21	7.43	1.054	5400
MVV039	POST	1.376	0.509	0.138	4.853	49.04	10.10	0.985	5060
MVV058	PRE	1.613	0.579	0.395	4.785	42.28	8.84	1.164	3400
MVV058	POST	1.305	0.528	0.348	4.627	39.98	8.64	1.092	2700
MVV100	PRE	1.947	0.675	0.397	4.564	35.87	7.86	1.020	3730
MVV100	POST	1.936	0.652	0.367	4.435	42.85	9.66	1.496	4100
MVV130	PRE	1.855	0.464	0.429	4.026	28.26	7.02	1.208	3600
MVV130	POST	1.927	0.536	0.392	3.667	31.29	8.53	1.258	2370

Appendix 9.5

Sheep ID	Time Point	pH	$PaCO_2$	PaO_2	HCO_3	tCO_2	BEvt	O2sat	BP	HR	RL
			kPa	kPa	mmol/l	mmol/l		%	mmHg	BPM	kPa/l/s
CON089	PRE	7.54	3.87	12.00	27.3	24.1	3.1	97.5	130	148	0.517
CON089	POST	7.53	4.13	8.00	27.9	25.7	4	92.4	129	125	0.571
CON091	PRE	7.59	3.73	14.40	30.1	26.1	6.2	98.5	123	108	0.430
CON091	POST	7.6	3.73	14.13	31.1	27	7.2	98.5	117	102	0.479
CON092	PRE	7.52	5.20	18.26	31.7	31.1	7.9	98.9	143	106	0.767
CON092	POST	7.52	5.60	5.87	33.4	33.9	10.1	80.9	135	83	0.869
CON094	PRE	7.6	4.27	13.86	32.7	29.7	8.9	98.3	118	120	0.619
CON094	POST	7.67	3.73	12.26	35	30.5	11.3	98	117	99	0.676
CON097	PRE	7.48	5.33	9.86	30.4	30.9	6.6	95.6	128	122	0.660
CON097	POST	7.51	4.93	8.26	31.4	31.1	7.7	93.9	122	83	0.633
MVV040	PRE	7.51	4.67	9.60	29.6	28.7	5.7	95.8	131	142	0.537
MVV040	POST	7.55	4.53	8.26	31.9	30.7	8.2	94.3	124	172	1.074
MVV004	PRE	7.47	5.60	11.73	31.2	31.7	7.4	96.9	117	102	0.709
MVV004	POST	7.47	6.13	17.86	33.4	34.3	9.7	98.8	94	80	0.698
MVV021	PRE	7.5	4.67	11.46	29	27.9	5	96.9	128	228	0.545
MVV021	POST	7.55	4.40	8.00	31	29.1	7.3	92.8	119	236	0.658
MVV026	PRE	7.45	4.80	12.13	26.5	25.5	2.3	97	126	209	0.639
MVV026	POST	7.54	4.13	8.00	29.7	27	5.9	92.6	121	241	0.849
MVV039	PRE	7.54	4.00	13.60	28.7	26.6	4.6	98.3	106	43	0.534
MVV039	POST	7.57	3.87	10.00	30	27.5	6.2	96.8	111	32	0.513
MVV058	PRE	7.5	5.07	10.53	29.7	29.3	5.8	95.8	121	114	0.510
MVV058	POST	7.56	4.40	11.33	32.6	30.6	8.9	97.2	118	86	0.525
MVV100	PRE	7.48	4.40	11.60	26.4	24.8	2.1	96.8	115	96	0.441
MVV100	POST	7.54	4.00	8.40	29.1	26.5	5.2	93.2	106	112	0.453
MVV130	PRE	7.5	4.53	14.00	28.6	26.6	4.6	98	117	191	0.538
MVV130	POST	7.52	4.53	11.20	29.8	27.6	1.2	96.6	113	221	0.727

Appendix 9.6

Appendix 9.7 Results of Mann-Whitney U tests used to compare the percentage changes in cardiorespiratory functional indices developed by seronegative and seropositive sheep. Significant differences are indicated in bold.

Variable	Mann-Whitney U test statistic	P
K	17.000	0.661
A/Vmax	17.000	0.661
E_{oes}	19.000	0.884
$V_{A,eff}$	26.000	0.380
$T_{L,CO,'sb'}$	**6.000**	**0.040**
$T_{L/VA}$	**5.000**	**0.028**
R_{int}	14.000	0.380
Cst	18.000	0.770
pH	11.500	0.213
$PaCO_2$	25.500	0.420
PaO_2	18.000	0.770
HCO_3	**5.000**	**0.028**
tCO_2	14.000	0.380
BEvt	**1.000**	**0.005**
O_2sat	15.000	0.464
BP	25.000	0.464
HR	11.000	0.188
C_{dyn}	32.000	0.079
R_L	16.000	0.558

Appendix 9.8 (overleaf) Summary statistics from microscopic point counting using a 121 point grid to estimate the tissue volume fraction (V_{vt}) and volume fraction of α-smooth actin ($V_{v'ASMA'}$) expressing tissue in lung parenchyma. The meaning and relevance of the statistical indices are fully explained in appendix 8.1.1..

Appendix 9.8

V_{vt}	MVV004	MVV021	MVV026	MVV039	MVV058	CON097	MVV100	MVV130
No of fields	120	100	90	90	81	180	170	100
Minimum	13.223	9.917	6.612	11.57	7.438	6.612	6.612	5.785
Maximum	95.041	56.198	56.198	73.554	58.678	39.669	39.669	48.76
Mean	27.355	26.041	21.368	22.507	29.681	18.54	15.989	18.545
Standard deviation	10.194	8.111	7.23	7.877	8.845	5.026	5.051	6.622
Standard error	0.931	0.811	0.762	0.83	0.983	0.375	0.387	0.662
Compound S.E.	0.043	0.046	0.044	0.045	0.055	0.029	0.028	0.039
Relative S.E.	0.158	0.178	0.207	0.202	0.185	0.157	0.174	0.211
Skewness (G1)	3.262	1.535	1.355	3.376	0.786	0.503	1.341	1.954
C.V.	0.373	0.311	0.338	0.35	0.298	0.271	0.316	0.357
Median	24.793	25.62	20.661	21.488	27.273	17.769	15.702	17.355
V_{vt}'ASMA'	MVV004	MVV021	MVV026	MVV039	MVV058	CON097	MVV100	MVV130
No of fields	120	100	90	90	81	180	170	100
Minimum	0	0	0	0	0	0	0	0
Maximum	7.438	11.57	9.917	10.744	10.744	9.917	7.438	9.917
Mean	2.734	3.488	3.618	3.774	3.836	1.809	1.988	3.388
Standard deviation	1.69	2.313	2.053	2.216	2.309	1.487	1.527	2.067
Standard error	0.154	0.231	0.216	0.234	0.257	0.111	0.117	0.207
Compound S.E.	0.014	0.017	0.018	0.019	0.02	0.009	0.01	0.017
Relative S.E.	0.502	0.487	0.504	0.493	0.516	0.504	0.494	0.494
Skewness (G1)	0.704	1.032	0.593	0.634	0.812	1.498	1.177	0.556
C.V.	0.618	0.663	0.567	0.587	0.602	0.822	0.768	0.61
Median	2.479	2.479	3.306	3.306	3.306	1.653	1.653	3.306

Sheep ID	Time Point	K	E_{oes}	$V_{A,eff}$	$T_{L,CO,'sb'}$	$T_{L/VA}$	R_{int}	Cst	BP	R_L	C_{dyn}
			kPa/ml	l	mmol/min/kPa	mmol/min/kPa/l	kPa/l/s	l/kPa	mmHg	kPa/l/s	ml/kPa
MVV026	PRE	1.456	0.058	3.88	30.28	7.81	1.088	3250	126	0.396	1660
MVV026	POST	1.467	0.063	3.91	24.45	6.25	1.135	3550	111	0.393	1170
MVV130	PRE	1.439	0.055	3.24	20.65	6.38	1.447	2450	120	0.692	1080
MVV130	POST	1.489	0.079	3.18	20.35	6.40	2.894	2650	116	0.775	550
MVV058	PRE	1.245	0.071	4.17	40.71	9.76	1.029	2780	113	0.463	1220
MVV058	POST	1.379	0.071	4.20	33.19	7.91	1.082	2900	106	0.380	1100
MVV039	PRE	1.516	0.033	5.17	37.58	7.27	0.912	3900	119	0.300	2860
MVV039	POST	ND	0.032	3.01	11.07	3.68	0.871	ND	104	0.286	3130
MVV021	PRE	0.896	0.105	2.81	19.27	6.85	1.235	1450	0	0.404	880
MVV021	POST	1.074	0.101	3.83	23.30	6.08	1.024	2100	0	0.381	960
CON097	PRE	1.063	ND	4.95	27.83	5.62	0.876	2800	125	0.395	2610
CON097	POST	1.489	0.058	4.79	27.25	5.69	0.971	2700	122	0.392	2310
MVV100	PRE	2.099	0.043	4.70	29.60	6.29	0.782	4100	109	0.235	2680
MVV100	POST	2.341	0.050	4.59	37.72	8.22	0.747	4450	99	0.285	1520

Appendix 9.9 Results from off-line (K, E_{oes}, $V_{A,eff}$, $T_{L,CO,'sb'}$, $T_{L/VA}$, R_{int} and Cst) and on-line (BP, R_L and C_{dyn}) analysis of data collected just before (PRE) and immediately after (POST) a 5 minute intravenous injection of clenbuterol (0.8μg/kg).

Sheep ID	Time Point	pH	$PaCO_2$	PaO_2	HCO_3	tCO_2	BEvt	O2sat
			kPa	kPa	mmol/l	mmol/l		%
MVV026	PRE	7.53	4.27	12.00	29.3	27.4	5.4	97.7
MVV026	POST	7.55	4.27	10.66	30.5	28.5	6.6	97.1
MVV130	PRE	7.52	4.53	18.00	30	28.8	6.1	98.9
MVV130	POST	7.53	4.53	10.40	31.3	30.1	7.5	96.8
MVV058	PRE	7.58	4.27	13.20	32.5	30.5	8.7	98.3
MVV058	POST	7.60	4.13	11.73	33.3	30.9	9.6	97.9
MVV039	PRE	7.51	4.40	15.60	28.7	27.5	4.7	98.6
MVV039	POST	7.49	4.67	14.80	27.9	27.2	3.8	98.4
MVV021	PRE	7.50	5.07	17.60	30.8	30.6	6.9	98.9
MVV021	POST	7.53	4.93	10.66	32.2	31.6	8.4	96.8
CON097	PRE	7.48	4.53	12.80	27.4	26.8	3.3	97.8
CON097	POST	7.48	4.67	12.13	27.7	27.3	3.6	97.5
MVV100	PRE	7.53	4.13	14.53	28.1	26.4	4	98.5
MVV100	POST	7.54	4.13	17.20	28.9	27.1	4.9	98.9

Appendix 9.10 Results from arterial blood gas and acid-base analysis of samples taken just before (PRE) and immediately after (POST) a 5 minute intravenous injection of clenbuterol (0.8μg/kg).

Appendix 9.11 The flow of gas across a membrane is determined by the pressure gradient for the gas across the membrane, the solubility of the gas within the membrane and the thickness and the area of the membrane (West, 1987). Assuming the driving pressure, the gas solubility and the alveolar capillary distance remains constant the 15% (median increase for seropositive sheep) increase in $T_{L,CO,'sb'}$ must represent a 15% increase in surface area. If a linear relationship between capillary surface area and volume is assumed (the ratio of pulmonary capillary blood volume to surface area in humans is approximately 0.58 ml/m^2)(calculated from Gehr et al. (1978)) then a 15 % increase in surface area could be accounted for by only a 15 % increase in capillary blood volume. There is approximately 200mls of pulmonary capillary blood in human lungs (Weibel, 1991) therefore an increase of 15% would only represent 30ml. This volume change is several orders of magnitude less than the noted changes in $V_{A,eff}$ for seropositive sheep.

PUBLICATIONS

Publications directly arising from this thesis include the following:

Collie,D.D.S., Watt,N.J., Warren,P.M., Begara,I., Luján,L.. (1993) Lung compliance, lung volume and transfer factor for carbon monoxide in anaesthetized sheep: normal values and reproducibility of measurements. Research In Veterinary Science, 55, pp137-143

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Lung compliance, lung volume and transfer factor for carbon monoxide in anaesthetised sheep: normal values and reproducibility of measurements

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Measurements of quasistatic compliance (C_{qst}), effective alveolar volume ($V_{A,eff}$) and single-breath transfer factor for carbon monoxide ($T_{L,CO,\ 'sb'}$) were completed in 16 normal, anaesthetised, adult Texel ewes. Regression equations were computed for these variables as a function of bodyweight and the optimal equations selected. The 95 per cent prediction intervals for the equations were calculated such that normal lung function in similar sheep could be accurately predicted. The long term reproducibility of these measurements was assessed in nine sheep, measured at monthly intervals over a period of five months. Although measurements made in individual sheep were often highly variable, the variation between repeated measurements on the separate days for the group was insignificant.

IN human respiratory research, lung function data are frequently assessed in relation to predicted values for the subject under study. These predicted values are generated using multiple regression equations previously calculated for a population similar to that from which the subject was obtained. The best reference (independent) variables used for humans are age, sex, stature and ethnic group, with these variables accounting for 60 per cent of the total variability about the regression lines (Cotes 1979). In comparative respiratory research, Stahl (1967) demonstrated, using data available from the literature, the relationship of several respiratory variables to bodyweight over a wide range of mammalian species. Such predictions, based on values over such a large weight range, may not agree with studies on smaller groups of animals of a particular species (Stahl 1967) where social and environmental factors may lead to physiological adaptation. Indeed in humans, bodyweight is not the anthropometric variable of choice due to large differences within populations regarding the contribution of adipose tissue and muscle in individuals (Cotes 1979).

In sheep, where breed, age, sex and environmental and social factors may influence body condition and hence bodyweight, reference to such predictions of respiratory variables based solely on bodyweight should be cautious. For this reason, where the study of respiratory variables in a particular category of sheep is planned, predicted normal values should ideally be generated using normal sheep of similar description.

Certain routine pulmonary function tests applied to humans can show considerable intra-subject variability (Guyatt et al 1975, Hutchison et al 1981, Pennock et al 1981) and similar variability is seen when comparable lung function measurements are applied to trained conscious sheep (Begin et al 1981) and cattle (Gallivan and McDonell 1988). Although this variability can preclude the use of these measurements to monitor individual animals over time, the use of groups of animals can overcome this limitation (Gallivan and McDonell 1988).

Whether for ethical or procedural reasons, circumstances may dictate that lung function measurements cannot be undertaken in naive conscious animals and in these situations anaesthesia is a frequently considered alternative. However, Southorn et al (1980) demonstrated that anaes-

thesia can contribute to intrasubject variability of pulmonary mechanics and static lung volume measurements in dogs. This variable effect of anaesthesia on pulmonary function measurements in individuals will presumably occur in other species and may limit their potential for monitoring changes in lung function in groups of animals over time.

The purposes of this study were, first, to generate regression equations relating pulmonary function variables to bodyweight for anaesthetised adult Texel sheep such that normal values could in future be generated for sheep of similar breed, age and sex, managed under similar conditions, and, secondly, to examine the long term reproducibility of lung function measurements obtained during anaesthesia.

Materials and methods

Animals

Sixteen adult Texel ewes (bodyweight 52 to 87 kg) were used. All the animals were free from clinically apparent cardiopulmonary dysfunction. Additional examinations including routine haematology, thoracic radiography, faecal examination for lungworm larvae and serological testing for presence of maedi-visna virus infection, confirmed the absence of significant cardiopulmonary dysfunction. The sheep were housed during the period of the measurements.

Anaesthesia

Food was withheld for 12 hours before anaesthesia which was achieved by intravenous administration of a single bolus of thiopentone sodium at a dose rate of 20 mg kg^{-1} bodyweight. The sheep were weighed on the morning of the procedure. Following induction the sheep were intubated using cuffed endotracheal tubes (diameter 9·5 to 10·5 mm) and placed in sternal recumbency with the head supported on a cushioned rest. They were ventilated with medical air (BOC) using a mechanical ventilator (Manley MN2, Hutchinson Blease) adjusted to maintain a tidal volume of 10 ml kg^{-1} bodyweight and respiratory rate of 10 breaths min^{-1}. To facilitate the measurement of respiratory flows a heated pneumotachograph (Fleisch number 2, Linton Instrumentation) was connected to the end of the endotracheal tube and the pressure drop across the pneumotachograph was measured using a sensitive differential pressure transducer (CS9, Mercury Electronics) with the signal subsequently integrated to yield respiratory volumes. Changes in transpulmonary pressure ($\triangle P_{tp}$) were measured using a differential pressure transducer (CS9, Mercury Electronics). One side of this transducer was connected via a polyethylene catheter (3 mm inner diameter, 4·5 mm outer diameter) to a latex balloon (length 15 cm, diameter 1·5 cm) sealed over the distal end of the catheter and placed in the caudal third of the thoracic oesophagus. The balloon was then evacuated and 2 ml of air was added. This volume was shown to be within the range of high compliance of this pressure recording system and was close to the minimal relaxed volume of the balloon in air. The other side of this transducer was connected to a side port perpendicular to the airway opening.

Outputs from pressure and flow measuring devices were recorded on a strip chart recorder (Linseis L6514, Belmont Instruments). Before measurements were made, the pressure recording system was calibrated against a water manometer and the integrated volume recording was calibrated using a 3 litre syringe.

Lung function measurements

Quasistatic compliance (C_{qst}). Following cessation of spontaneous respiratory efforts (within two to three minutes of commencing mechanical ventilation in every case) the sheep were disconnected from the ventilator and allowed to exhale passively to functional residual capacity (FRC). The lungs were then actively inflated to $\triangle P_{tp}$= 3 kPa using the 3 litre calibrated syringe filled with room air and immediately thereafter allowed to deflate passively to FRC. This procedure was repeated twice to stabilise lung volume history before measurements of quasistatic compliance were made. The volume of the lungs at $\triangle P_{tp}$ = 3 kPa was defined as total lung capacity (TLC). To measure C_{qst}, the lungs were again inflated to TLC, however the passive exhalation was interrupted in a stepwise fashion by occluding the airway opening eight to 15 times for two to three second intervals. By plotting $\triangle P_{tp}$ against expired volume a quasistatic deflation curve was obtained. The slope of the linear portion of the curve from FRC to 40 per cent of TLC was determined by least squares linear regression and this value taken as C_{qst}, expressed in units of litres kPa^{-1}. To achieve adequate data

points for the least squares regression, the measurement procedure outlined above was repeated twice with pooling of data points such that a single 'within procedure' value of C_{qst} for each sheep was obtained.

Effective alveolar volume ($V_{A,eff}$) and transfer factor for carbon monoxide ($T_{L,CO}$)

$V_{A,eff}$, using a single-breath helium dilution technique, and $T_{L.CO}$, using a forced single-breathhold technique, were measured concurrently as part of the same procedure. Following a period of mechanical ventilation the sheep were disconnected from the ventilator and allowed to exale passively to FRC. The 3 litre calibrated syringe was then used to inflate the lungs to $\triangle P_{tp}$ = 3 kPa with a carbon monoxide and helium in air gas mixture (4 per cent helium, 0·3 per cent carbon monoxide, 85·7 per cent air; BOC). A valve at the airway opening was closed and this gas mixture was held in the lungs for a period of 10 to 12 seconds. The valve was then opened momentarily to allow wash out of dead space before the remaining gas in the lungs was collected in an evacuated rebreathing bag. The concentration of helium in this expired alveolar gas sample was measured using a katharometer (Resparameter Mk 4, P. K. Morgan) and $V_{A,eff}$ in litres, that is, the alveolar volume at which the breath was held minus the dead space of the airways and equipment was calculated as follows:

$$V_{A.eff} = 1{\cdot}05 \,.\, (V_{syr} - V_{D.an} - V_{D.eq}) \,.\, (He_I/He_E)$$

where V_{syr} = volume used to inflate lungs to $\triangle P_{tp}$ = 3 kPa, $V_{D.an}$ = anatomical dead space, $V_{D.eq}$ = instrumental dead space, He_I = helium concentration in the syringe and He_E = helium concentration in the expired sample (Quanjer et al 1983). Instrumental dead space was calculated from internal dimensions and the anatomical dead space was estimated using the equations of Stahl (1967) which relate respiratory variables to bodyweight in a range of species.

Single-breath transfer factor for carbon monoxide ($T_{L.CO,'sb'}$) was calculated as follows:

$$T_{L.CO,'sb'} = 53{\cdot}6 \,.\, V_{A.eff} \,.\, t^{-1} \,.\, \log_{10}(CO_I.He_E.CO_E^{-1}.He_I^{-1})$$

where t = time of breathholding in seconds and He_E and CO_E were alveolar concentrations, and He_I and CO_I syringe concentrations of helium and carbon monoxide, respectively (Cotes 1983). Time of breathholding was calculated according to the recommendations of the Epidemiology Standardisation Project (ESP) (Ferris 1978). A blood sample was taken during the period of anaesthesia and haemoglobin concentration measured using an automated analyser. $T_{L.CO,'sb'}$ values were then adjusted to a standard haemoglobin concentration of 14·6 g dl^{-1} according to the method of Cotes (1979). $T_{L.CO,'sb'}$ is expressed in units of mmol min^{-1} kPa^{-1}. Since the transfer factor is positively correlated with the lung volume at which the measurement is made, it was also expressed per litre of alveolar volume, that is, T_L/V_A. The mean value of three determinations of $V_{A,eff}$ and $T_{L.CO,'sb'}$ was calculated and used in the statistical analyses. The average duration of the completed series of measurements was approximately 20 minutes.

Experimental design and statistical analysis

Long term reproducibility of the lung function data was examined by repeating measurements in the same group of nine sheep at monthly intervals over a period of five months. A two-way mixed model analysis of variance (ANOVA) without replication was used to estimate the between day variability of lung function variables for the group. In this analysis, the individual sheep are considered the random factor and the time dimension a fixed treatment effect (Sokal and Rohlf 1981). A Kolmogorov-Smirnov test was used to test for normal distribution of the pooled error terms in each ANOVA and a Bartlett's test was used to check for homogeneity of variances between samples in each ANOVA (Sokal and Rohlf 1981). Data from each ANOVA satisfied both of the above preconditions. The coefficient of variation (CV) for the repeated measurements of each variable in individual sheep was also calculated.

The relationship of individual lung function variables to bodyweight was examined for all 16 sheep. Lung function and bodyweight measurements were made at least three times on each sheep with the interval between measurements being between 30 and 77 days. The data from a total of 70 procedures were used in the analysis. Regression analyses based on linear, polynomial and allometric regression equations were executed to determine the relationship between bodyweight (the independent variable) and the lung function data (the dependent variable). Using the most statistically significant regression equations, 95 per

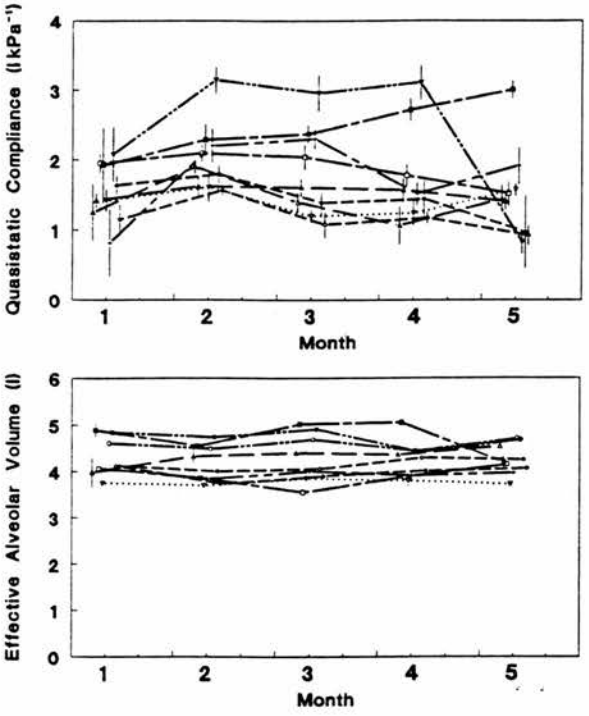


FIG 1: Long term variability of C_{qst} (litres kPa^{-1}) and $V_{A,eff}$ (litre^{-1}) measurements made in nine sheep at monthly intervals over a period of five months. Error bars in the upper graph represent the 95 per cent confidence limits for the slope of the regression line between pressure and volume measurements, that is, the compliance. Error bars in the lower graph represent ± SD

cent prediction intervals for individual lung function variables were calculated over the range of bodyweights studied.

Results

Long term variabilities of C_{qst} and $V_{A.eff}$ are illustrated in Fig 1. Coefficients of variation for repeated measurements of each variable in individual sheep are illustrated in Fig 2. The average intrasubject CV for the repeated measurements was 18·8 ± 9·23 (mean ± standard deviation) for C_{qst}, 10·3 ± 4·45 for $T_{L.CO.'sb'}$, 9·8 ± 4·02 for T_L/V_A and 3·4 ± 1·71 for $V_{A.eff}$. The results of the analyses of variance are shown in Table 1. There were no

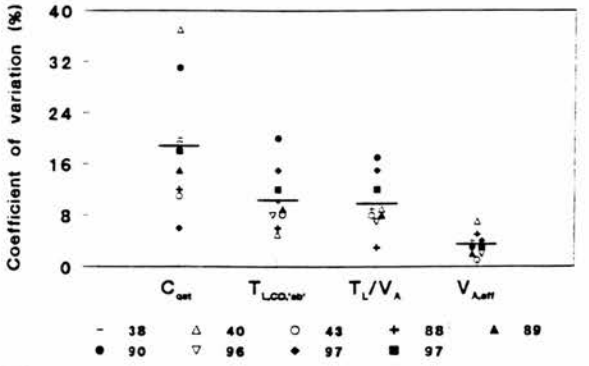
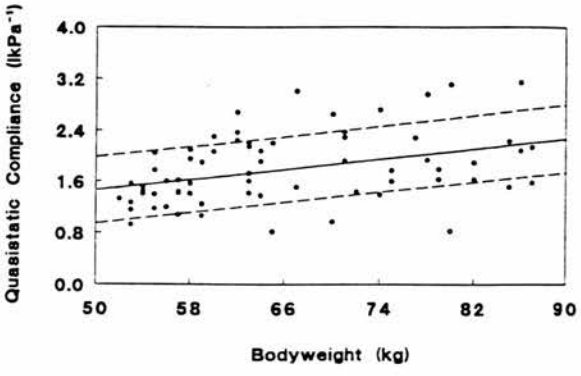


FIG 2: Individual coefficients of variation for repeated measurements of C_{qst}, $V_{A.eff}$, $T_{L.CO.'sb'}$ and T_L/V_A made in nine sheep at monthly intervals over a period of five months. The horizontal bars represent the mean values

TABLE 1: Results of analysis of variance for repeated C_{qst}, $V_{A,eff}$, $T_{L,CO,'sb'}$ and T_L/V_A measurements made in nine sheep at monthly intervals over a period of five months

	Between days		Between sheep	
	F ratio	Significance	F ratio	Significance
C_{qst}	2·32	NS	5·77	***
$V_{A,eff}$	1·33	NS	19·94	***
$T_{L.CO.'sb'}$	2·60	NS	12·77	***
T_L/V_A	2·15	NS	11·31	***

For all variables there was no significant differences between days. Differences between sheep were highly significant. NS Not significant, P>0·05; *** P<0·001



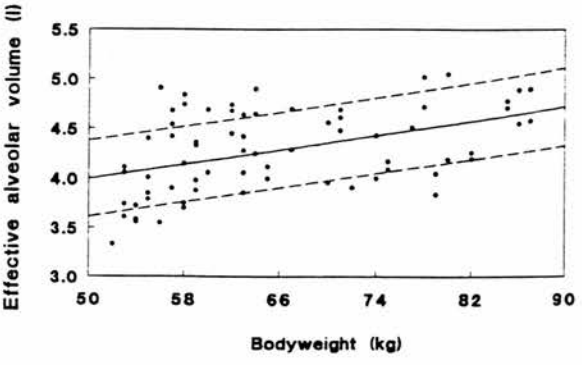


FIG 3: Relationship between of C_{qst} (litres kPa^{-1}) and $V_{A.eff}$ (litre^{-1}) and bodyweight (kg). Repeated measurements were made in 16 sheep. Solid lines depict the regression equations that best represent the relationship between the dependent variables and bodyweight. Dotted lines represent the 95 per cent prediction intervals for the dependent variables in individual subjects

TABLE 2: Selected regression equations relating C_{qst} (litres kPa^{-1}), $V_{A,eff}$ (litre^{-1}), $T_{L,CO,'sb'}$ (mmol min^{-1} kPa^{-1}) and T_L/V_A (mmol min^{-1} kPa^{-1} litre^{-1}) to bodyweight (kg)

Variable	Unit	Regression equation	F	r^2 (%)	P
C_{qst}	(litres kPa^{-1})	0·460+0·0201xBW	11·72	14·9	0·001
$V_{A,eff}$	(litre^{-1})	3·09+0·0181xBW	17·53	20·7	<0·001
$T_{L,CO,'sb'}$	(mmol min^{-1} kPa^{-1})	3·27+0·110xBW	40·79	37·8	<0·001
T_L/V_A	(mmol min^{-1} kPa^{-1} litre^{-1})	1·52+0·0143xBW	10·47	13·5	<0·005

F Variance ratio, r^2 Determination coefficient (expressed as a percentage), P Degree of significance of the variance ratio

significant differences between days for the group for any of the lung function variables although between sheep differences were highly significant.

A significant relationship was found between all of the measured variables and bodyweight. These relationships are illustrated in Figs 3 and 4. The most significant regression equations were linear for all variables and these equations, together with the variance ratio (F), the determination coefficient (r^2) and degree of significance of the variance ratio are shown in Table 2.

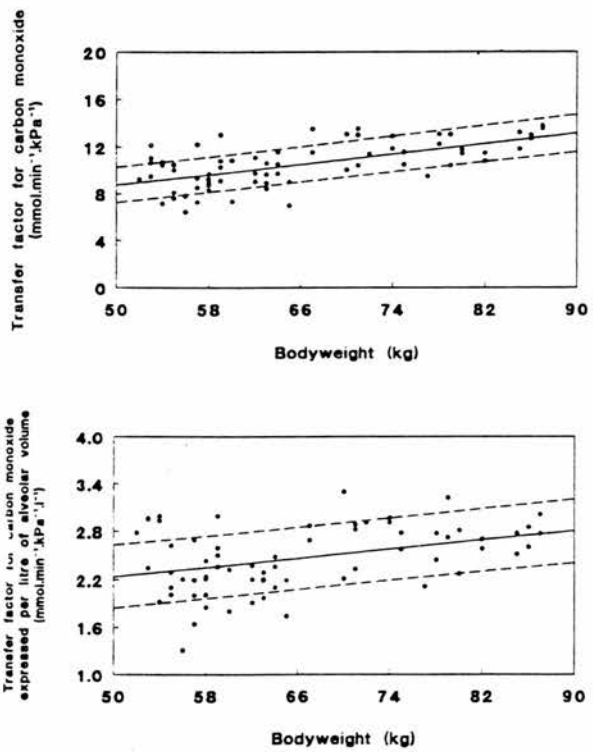


FIG 4: Relationship between $T_{L,CO,'sb'}$ (mmol min^{-1} kPa^{-1}) and T_L/V_A (mmol min^{-1} kPa^{-1} litre^{-1}) and bodyweight (kg). Repeated measurements were made in 16 sheep. Solid lines depict the regression equations that best represent the relationship between the dependent variables and bodyweight. Dotted lines represent the 95 per cent prediction intervals for the dependent variables in individual subjects

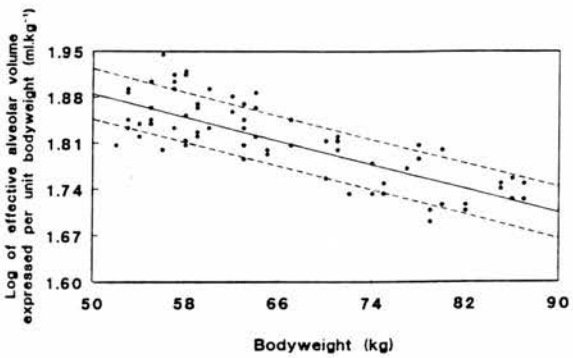


FIG 5: Relationship between log $sV_{A,eff}$ (ml kg^{-1}) and bodyweight. Repeated measurements were made in 16 sheep. Solid lines depict the regression equation that best represents the relationship between the dependent variable and bodyweight. Dotted lines represent the 95 per cent prediction intervals for the dependent variable in individual subjects

Discussion

The predictions of Stahl (1967) encompassed the variables measured in this study and these variables were shown to be positively correlated with bodyweight. For mammals within the range of weights studied in this paper (52 to 87 kg), Stahl's (mean) predictions are 1·53 to 2·66 litre kPa^{-1} for lung compliance, 6·67 to 11·99 mmol min^{-1} kPa^{-1} for diffusing capacity, 1·876 to 1·976 mmol min^{-1} kPa^{-1} litre^{-1} for diffusing capacity/TLC and 3·5 to 6·0 litres for total lung capacity.

Values of $V_{A,eff}$ and C_{qst} for the heavier bodyweight sheep were lower than Stahl's predictions and the converse was true for T_L/V_A, that is, measured values for the heavier sheep were greater than predicted. The latter finding is presumably a reflection of the reduction in $V_{A,eff}$. One explanation for these anomalies would be that rather than reflecting an increase in skeletal dimensions, greater weight in these sheep reflects an increase in adipose and perhaps muscle tissue. That this is indeed likely is shown by the reduction in specific effective alveolar volume ($sV_{A,eff}$), that is, alveolar volume expressed per unit bodyweight, with

increasing bodyweight in these sheep (Fig 5). The ratios of various lung volumes to bodyweight are relatively constant and independent of size, that is, they do not change appreciably between species (Stahl 1967). The reduction in $sV_{A,eff}$ with increasing bodyweight indicates that overfeeding sheep can deleteriously alter the normally optimal relationship between body size and lung functional indices. This is a recognised concept in humans (Schoenberg et al 1978) where increased muscularity or obesity have opposite effects on ventilatory function. The above hypothesis does not, however, explain the agreement between measured and predicted $T_{L.CO.'sb'}$. As is thought to be the case in dogs (Robinson et al 1972), $T_{L.CO.'sb'}$ in sheep may be related to metabolic bodyweight rather than bodyweight per se.

One previous study of respiratory variables in anaesthetised sheep of bodyweight 42·6 ± 4·7 kg (mean ± SD) reported a value of dynamic lung compliance of 1·08 ± 0·316 litre kPa^{-1} (mean ± SD) (Halmagyi and Colebatch 1961). This low value may simply be a reflection of the method of anaesthetic maintenance they used. In their study no mechanical ventilation was used during anaesthesia, and, therefore, progressive atelectasis of dependent lung units and reduced lung compliance was a likely consequence. In the present study, thiopentone was administered as a single bolus dose, and so the level of anaesthesia would have varied during the procedure. As a consequence, static lung volumes and mechanical and gas exchange properties of the lung would also have varied (Southorn et al 1980). This variation will necessarily be included in the measurements made, however, the adoption of a standardised dosage regime and procedural protocol will have helped to minimise such between procedure variation.

The observed regression equations relating bodyweight to lung function variables should prove valuable in predicting normal values for sheep of similar breed, age, sex, weight and conformation to those used in the present study and studied under identical conditions. The 95 per cent prediction intervals were calculated using the estimated standard deviation of the dependent variables over the range of weights studied (Gardner and Altman 1989). These intervals serve to define the limits of normality for measurements of pulmonary function variables made in sheep with similar characteristics to those studied. The use of the standard deviation as a measure of variability within a population is appropriate where the data are homoscedastic, that is, the variation is independent of the magnitude of the value measured. There are, however, inadequate data points collected in this study to determine accurately whether the data are homo- or heteroscedastic.

A knowledge of the intrasubject variation over a period of time is essential if time sequential changes in pulmonary function values for individuals are to be accurately interpreted. In human respiratory research, data are often treated as heteroscedastic and reproducibility is expressed in terms of the CV. By expressing the long term variability for repeated measurements in terms of the CV, knowledge of what would constitute a significant change in an individual's lung function can be gained. Given the calculated average CVs for repeated measurements in normal anaesthetised sheep (Fig 2), it can be predicted (Pennock et al 1981) that significant ($P<0·05$) month-to-month changes are approximately 30·8 per cent in the C_{qst}, 16·9 per cent in the $T_{L.CO.'sb'}$, 16·1 per cent in the T_L/V_A and 5·6 per cent in the $V_{A,eff}$ measurements. Previous reports have demonstrated similar intrasubject variability of lung function measurements in humans (Guyatt et al 1975, Hutchison et al 1981), ponies (Derksen et al 1982) and cattle (Gallivan and McDonell 1988) and illustrate the problems of interpreting lung function in individuals over time.

For all of the measured variables, the variation between repeated measurements on the separate days for the group was insignificant, whereas the variation between individual sheep was highly significant. Collective lung functional measurements on groups (n=9) of normal anaesthetised sheep do not therefore change significantly over time. Gallivan and McDonell (1988) in repeated analyses on four conscious adult cows found significant differences between days for the group for respiratory frequency and inspiratory and expiratory times and concluded that a control group should be used during any long-term study on small groups of animals.

Knowledge of both the predicted normal ranges for the variables C_{qst}, $V_{A,eff}$, $T_{L.CO.'sb'}$ and T_L/V_A, and awareness of the long term reproducibility of measurements of these variables in anaesthetised adult Texel sheep will allow more accurate interpretation, on a research basis, of respiratory disease in similar animals.

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Effects on lung compliance, lung volume, and single-breath transfer factor for carbon monoxide in sheep with lentivirus-induced lymphoid interstitial pneumonia

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Summary

Static lung compliance, static lung volumes, and transfer factor for carbon monoxide were measured in 12 anesthetized adult Texel ewes seropositive for maedi-visna virus (MVV) and in 11 breed-, sex-, and age-matched seronegative controls. Median static lung compliance in MVV-infected sheep (1.24 $L \cdot kPa^{-1}$; range, 0.27 to 2.20 $L \cdot kPa^{-1}$) was not significantly different from that in controls (1.58 $L \cdot kPa^{-1}$; range, 0.82 to 2.08 $L \cdot kPa^{-1}$). Median body weight of MVV-infected sheep (56 kg; range, 40 to 75 kg) was significantly ($P < 0.05$) less than that of controls (65 kg; range, 53 to 87 kg). Median effective alveolar lung volume in MVV-infected sheep (3.36 L; range, 1.44 to 4.52 L) was significantly ($P < 0.01$) less than that in controls (4.12 L; range, 3.75 to 4.90 L). Median effective end expiratory lung volume in MVV-infected sheep (1.20 L; range, 0.56 to 1.99 L) was significantly ($P < 0.001$) less than that of controls (1.98 L; range: 1.76 to 2.78 L). Median lung volumes expressed per unit of body weight did not differ significantly between the groups. Median single-breath transfer factor for carbon monoxide in MVV-infected sheep (7.89 $mmol \cdot min^{-1} \cdot kPa^{-1}$; range, 3.45 to 12.74 $mmol \cdot min^{-1} \cdot kPa^{-1}$) was significantly ($P < 0.001$) less than that in controls (14.10 $mmol \cdot min^{-1} \cdot kPa^{-1}$; range, 10.02 to 18.30 $mmol \cdot min^{-1} \cdot kPa^{-1}$). Median transfer factor expressed per liter of alveolar volume in MVV-infected sheep (2.44 $mmol \cdot min^{-1} \cdot kPa^{-1} \cdot L^{-1}$; range, 1.28 to 3.72 $mmol \cdot min^{-1} \cdot kPa^{-1} \cdot L^{-1}$) was significantly ($P < 0.05$) less than that in controls (3.22 $mmol \cdot min^{-1} \cdot kPa^{-1} \cdot L^{-1}$; range, 2.47 to 3.74 $mmol \cdot min^{-1} \cdot kPa^{-1} \cdot L^{-1}$). These findings indicate that static lung volumes and transfer factor for carbon monoxide are significantly decreased in adult sheep naturally infected with MVV.

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The principal pathologic feature of maedi-visna virus (MVV) infection in sheep is a slowly progressive lymphoid interstitial pneumonia[1] that causes dyspnea, reduced exercise tolerance, and loss of body condition.[2] The MVV shares considerable sequence homology and morphologic similarities with human immunodeficiency virus, also a lentivirus, the etiologic agent of the acquired immune deficiency syndrome (AIDS).[3] Lymphoid interstitial pneumonia (LIP) is a recognized pulmonary complication of AIDS in adults[4] and children[5]; however, the pathogenesis of LIP in people has yet to be elucidated, as have appropriate diagnostic and therapeutic measures.[5] It is likely that the primary lymphoproliferative response seen in association with MVV-infection and with AIDS is a result of similar immunologic dysregulation.[6] As a consequence, further investigation into the pathogenesis of MVV-infection is warranted.

Functional changes in human interstitial lung disorders are characterized by reduction in compliance, lung volumes, and transfer factor for carbon monoxide.[7,8] Physiologic assessment of these functional changes, in combination with other investigative techniques, such as radiography, have proved useful in quantifying and monitoring the progression of LIP associated with AIDS in human beings.[9,10]

Clinical signs of MVV infection are apparent only in the later stages of the natural disease, and little is known about disease progression in the preclinical phase. Meaningful investigation of the pathogenesis of naturally acquired maedi-visna requires the use of minimally invasive techniques to stage and monitor the course of disease. The use of physiologic techniques to measure lung function of infected sheep is a logical progression from clinical examination, which

tends to identify sheep only in the latter stages of the disease.

To the authors' knowledge, lung functional changes associated with MVV infection have not been described; however, if present, these changes may be of value for staging the disease in the preclinical phase. It was, therefore, considered appropriate to examine sheep with natural MVV infection to characterize the effect of this disease on static and gas exchange properties of the lungs.

Materials and Methods

Sheep—Twelve adult Texel ewes (median body weight, 55 kg; range, 40 to 75 kg), seropositive for MVV on the basis of results of the agar gel immunodiffusion test,[11] and 11 breed-, age-, and sex-matched controls (median, 65 kg; range, 53 to 87 kg), seronegative for MVV), were studied. Body weight median and range values reflect the poorer body condition of the seropositive sheep; however, both groups had similar skeletal conformation and were managed under similar conditions. The sheep had no evidence of respiratory tract disease unrelated to MVV infection as indicated by results of clinical examination, routine hematologic analysis, thoracic radiography, and fecal examination for lungworm larvae by use of a modified Baermann technique.[12]

Animal preparation and anesthesia—General anesthesia was induced by IV administration of thiopentone sodium (20 mg/kg of body weight). Sheep were intubated with cuffed endotracheal tubes (9.5 to 10.5 mm)[a] and positioned in sternal recumbency with the head supported on a cushioned rest. Esophageal pressure was measured by use of a latex balloon (length, 15 cm; diameter, 1.5 cm; thickness, 0.05 mm), which was sealed over the end of a polyethylene catheter (3 mm ID, 4.5 mm OD), which in turn had a number of holes cut in spiral manner in the end covered by the balloon. Prior to each series of measurements, the pressure-volume characteristics of the balloon-catheter assembly were validated according to the method of Senterre and Geubelle.[13] The balloon was then evacuated, and 2 ml of air was added. This volume was shown to be within the range of high compliance of this pressure recording system and was close to the minimal relaxed volume of the balloon in air. The free end of the esophageal catheter was connected to a differential pressure transducer,[b] and pressures were recorded on a strip chart recorder.[c] Prior to passing the balloon catheter assembly, the distance from the nares to the middle of the thoracic portion of the esophagus was visually approximated, and this length was marked on the catheter to serve as initial validation of balloon position after insertion. The balloon was passed via the mouth and advanced until tracheal artifacts were absent from the tracings,[14] negative deflections were observed in inspiration, and clearly defined cardiac oscillations were discernible in the waveform. These precautions ensured that the balloon was placed in the midthoracic portion of the esophagus where the influences of periesophageal structures are minimal in cattle and goats.[15,16] The other side of the differential pressure transducer was connected to a side port perpendicular to the airway opening, and the pressure thus recorded was defined as transpulmonary pressure (P_L). The pressure recording system was calibrated against a water manometer prior to each series of measurements.

If required, anesthesia was maintained, using an IV administered saline solution drip containing 0.20 to 0.25 mg of thiopentone sodium/kg infused at a rate of 2.0 to 2.5 ml/min.[17] Sheep were ventilated, using a mechanical ventilator[d] adjusted to maintain a tidal volume of 10 ml/kg and respiratory rate of 10 breaths/min.

Respiratory flow was measured, using a heated pneumotachograph[e] connected to the end of the endotracheal tube. The pressure decrease across the pneumotachograph was measured, using a sensitive differential pressure transducer, and the signal was integrated to yield respiratory volumes,[b] which were plotted on the chart recorder. The flow recording system was calibrated, using a 3-L syringe.

Static lung compliance (C_L)—After disconnecting the ventilator and allowing the sheep to passively expire to its relaxed functional residual capacity (FRC), the 3-L calibrated syringe was attached to the endotracheal tube and the lungs were inflated to P_L of 3 kPa. The volume of the lungs at P_L of 3 kPa was defined as total lung capacity (TLC). The sheep was then allowed to passively exhale to FRC; and the procedure was repeated twice to standardize the volume history of the lungs prior to measurements being made.[18] To measure C_L, the lungs were again inflated to P_L of 3 kPa; however, the passive expiration was interrupted in stepwise manner by occluding the airway opening 8 to 15 times for 2- to 3-second intervals. By plotting P_L against expired volume, a static deflation curve was obtained. The slope of the linear portion of the curve from FRC to 40% of the way from FRC to TLC was determined by use of least-squares linear regression, and this value was taken as C_L, expressed in units of $L \cdot kPa^{-1}$.

Lung volumes and transfer factor for carbon monoxide ($T_{L\ CO}$)—Lung volumes were measured, using a single-breath helium-dilution method performed in conjunction with the measurement of the transfer factor. A carbon monoxide and helium mixture (14% helium, 0.3% carbon monoxide, 85.7% air) was used to inflate the lungs from FRC to P_L of 3 kPa, at which point the airway opening was closed for 10 to 12 seconds. The valve was opened momentarily to allow washout of dead space, and thereafter, the expired alveolar gas was collected in an evacuated rebreathing bag. The concentration of helium in

[a] Blue Line, Portex, Kent, United Kingdom.
[b] CS9, Mercury Electronics, Glasgow, United Kingdom.
[c] Linseis L6514, Belmont Instruments, Glasgow, United Kingdom.
[d] Manley MN2, Hutchinson Blease, Chesham, Bucks, United Kingdom.
[e] Fleisch Ho. 2, Linton Instrumentation, Palgrave, Norfolk, United Kingdom.

Table 1—Means, medians, ranges, and coefficients of variation (CV) for pulmonary variables in control sheep (n = 11) and sheep with maedi-visna virus (MVV) infection (n = 12)

Measurement	Control				MVV-infected			
	Mean	Median	Range	CV (%)	Mean	Median	Range	CV (%)
Body weight (kg)	69.1	65.0	53 to 87	18	55.1	55.5*	40 to 75	15
C_L ($L \cdot kPa^{-1}$)	1.56	1.58	0.82 to 2.08	25	1.26	1.24	0.27 to 2.20	48
$V_{A\,eff}$ (L)	4.31	4.12	3.75 to 4.90	9	3.30	3.37*	1.44 to 4.52	26
$sV_{A\,eff}$ ($ml \cdot kg^{-1}$)	64	64	51 to 82	15	60	61	32 to 90	27
$V_{EEL\,eff}$ (L)	2.09	1.98	1.76 to 2.78	14	1.31	1.20†	0.56 to 1.99	34
$sV_{EEL\,eff}$ ($ml \cdot kg^{-1}$)	31	28	23 to 49	27	24	24	12 to 38	37
$T_{L\,CO\,sb}$ ($mmol \cdot min^{-1} \cdot kPa^{-1}$)	14.02	14.10	10.02 to 18.30	16	8.15	7.89†	3.45 to 12.74	38
T_L/V_A ($mmol \cdot min^{-1} \cdot kPa^{-1} \cdot L^{-1}$)	3.24	3.22	2.47 to 3.74	11	2.50	2.44‡	1.28 to 3.72	30

* Median value significantly ($P < 0.01$) different from value for control sheep. † Median value significantly ($P < 0.001$) different from value for control sheep. ‡ Median value significantly ($P < 0.05$) different from value for control sheep.

C_L = static lung compliance; $V_{A\,eff}$ = effective alveolar lung volume; $sV_{A\,eff}$ = specific effective alveolar lung volume (ie, $V_{A\,eff}$ expressed per unit of body weight); $V_{EEL\,eff}$ = end-expiratory lung volume; $sV_{EEL\,eff}$ = specific end-expiratory lung volume; $T_{L\,CO\,sb}$ = single-breath transfer factor for carbon monoxide; and T_L/V_A = transfer factor per liter of alveolar volume.

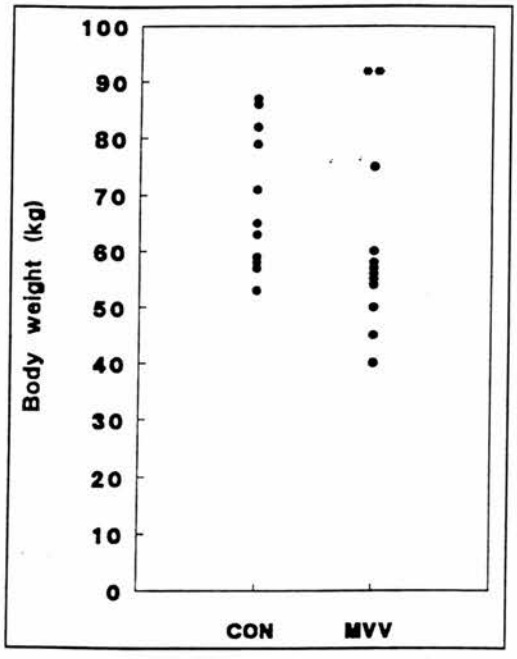

Figure 1—Body weight for 11 control (CON) and 12 maedi-visna virus (MVV)-infected sheep. ** Significantly ($P < 0.01$) lower than values for controls.

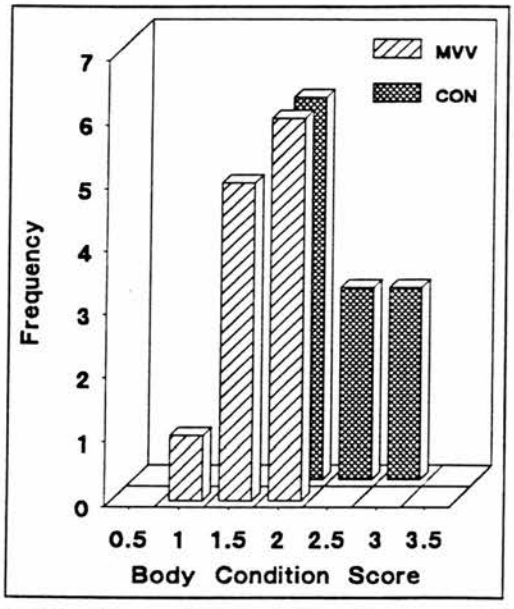

Figure 2—Histogram illustrating body condition scores for 11 CON and 12 MVV-infected sheep. Condition scores for the MVV-infected sheep were significantly ($P < 0.01$) less than the values for CON sheep.

the bag was determined, using a katharometer.[f] Dead space of equipment was estimated from internal dimensions, and anatomic dead space was estimated, using the appropriate allometric equation of Stahl,[19] which relates anatomic dead space to body weight.

The effective alveolar volume ($V_{A\,eff}$) in liters (ie, the alveolar volume at which the breath was held minus the dead space of the airways) was calculated as follows:

$$V_{A\,eff} = 1.05 \times (V_{syr} - V_{D\,an} - V_{D\,eq}) \times (He_I/He_E)$$

where V_{syr} = volume used to inflate lungs to P_L of 3 kPa, $V_{D\,an}$ = anatomic dead space, $V_{D\,eq}$ = instrumental dead space, He_I = helium concentration in the syringe, and He_E = helium concentration in the expired sample. The coefficient 1.05 is a factor used to account for a change in He_E attributable to carbon

[f] Resparameter Mk 4, PK Morgan, Chatham, Kent, United Kingdom.

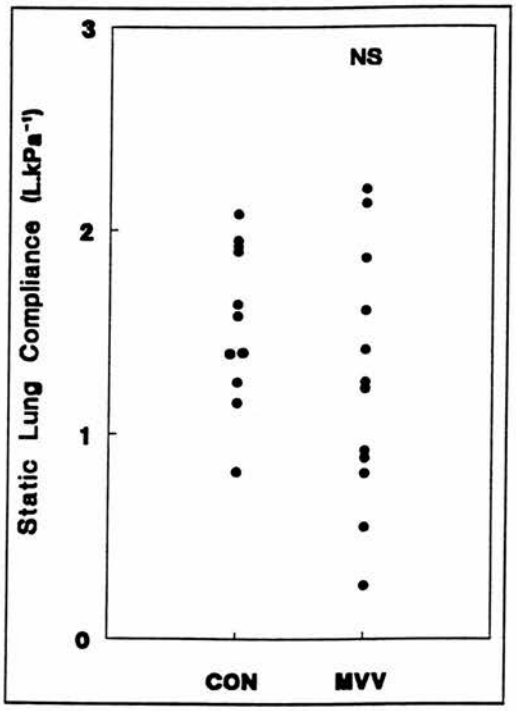

Figure 3—Static lung compliance values ($L \cdot kPa^{-1}$) for 11 CON and 12 MVV-infected sheep. NS = no significant difference between the groups.

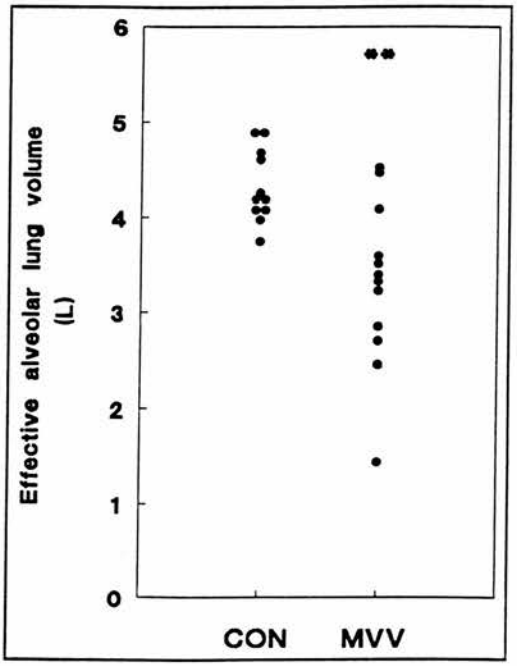

Figure 4—Effective alveolar lung volume values for 11 CON and 12 MVV-infected sheep. *See* Figure 1 for key.

dioxide absorption during breath-holding.[20] By subtracting V_{syr} from $V_{A\ eff}$, an estimate of the lung volume at the end-expiratory level ($V_{EEL\ eff}$) was obtained.

The single-breath transfer factor for carbon monoxide ($T_{L\ CO\ sb}$) was determined, using a modification of the technique described by Karp et al.[21] The technique involves 10-seconds of breath-holding of a carbon monoxide and helium in air gas mixture, with subsequent collection of an alveolar gas sample. The concentration of carbon monoxide in the syringe initially and in the alveolar gas sample, together with the time of breath-holding and the alveolar volume during breath-holding, are used in the calculation of $T_{L\ CO\ sb}$ as follows:

$$T_{L\ CO\ sb} = 53.6 \times V_{A\ eff} \times t^{-1} \times \log_{10} (CO_I \cdot He_E \cdot CO_E^{-1} \cdot He_I^{-1})$$

where t is the time of breath-holding in seconds, $V_{A\ eff}$ is the effective alveolar volume, and He_E and CO_E are end-tidal alveolar concentrations and He_I and CO_I are syringe concentrations of helium and carbon monoxide respectively.[22] Units of $T_{L\ CO\ sb}$ are expressed as $mmol \cdot min^{-1} \cdot kPa^{-1}$. Because the transfer factor is positively correlated with the lung volume at which the measurement is made, it is also expressed per liter of alveolar volume (ie, T_L/V_A).

During the period of anesthesia, a blood sample was collected and hemoglobin concentration was measured by use of an automated analyzer.[8] This concentration of hemoglobin was used in the equation of Cotes,[23] to adjust observed $T_{L\ CO\ sb}$ to a standard hemoglobin concentration of 14.6 g/dl.

Analysis of data—Three determinations of C_L lung volumes, and transfer factor were made on each sheep, and the mean value was calculated. Absolute lung volumes were also expressed per unit of body weight (ie, specific end expiratory lung volume [$sV_{EEL\ eff}$] and specific effective alveolar volume [$sV_{A\ eff}$]). The values recorded for the MVV-seropositive and -seronegative groups of sheep were statistically compared, using the Mann-Whitney test. Linear regression analysis with ANOVA was used to examine the relation between C_L and body weight and C_L and $V_{A\ eff}$ for each group.

Results

Mean, median, range, and coefficients of variation were determined for measurements of body weight, C_L, absolute and specific lung volumes, transfer factor, and transfer factor expressed per unit of alveolar volume for each group of sheep (Table 1).

The 95.5% confidence interval for the difference in population medians for body weight ranged from −26 to −3 kg. This difference was statistically significant ($P < 0.01$; Fig 1). Body condition scores for

[8] Baker System 9000, Serono Baker Diagnostics, Allentown, Pa.

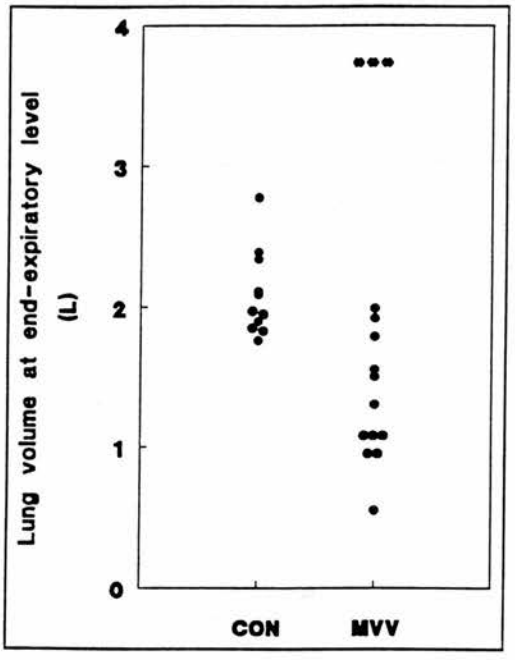

Figure 5—Lung volume values at end-expiratory level for 11 CON and 12 MVV-infected sheep. *** Significantly ($P < 0.001$) lower than values for controls.

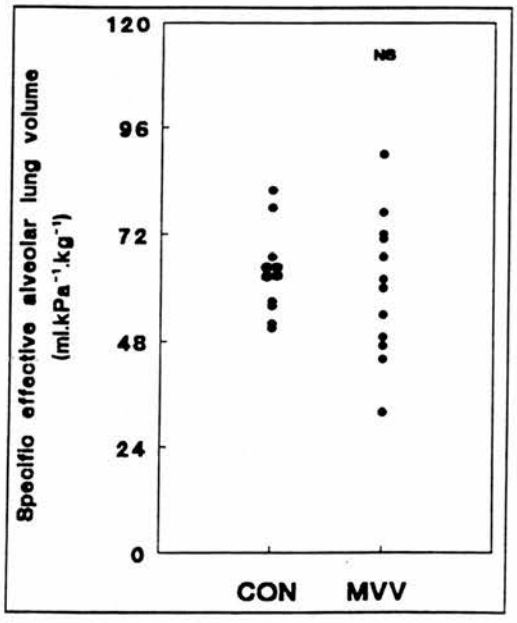

Figure 6—Specific effective alveolar lung volume values for 11 CON and 12 MVV-infected sheep. *See* Figure 3 for key.

MVV-infected sheep were significantly ($P < 0.01$) less than scores for controls (Fig 2).

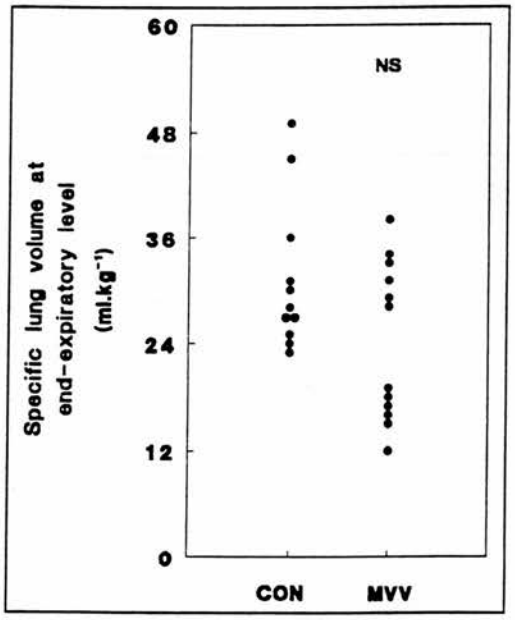

Figure 7—Specific end-expiratory level lung volume values for 11 CON and 12 MVV-infected sheep. *See* Figure 3 for key.

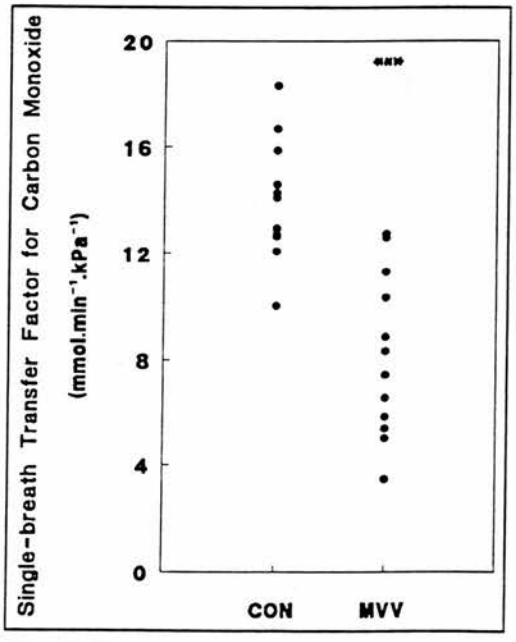

Figure 8—Single-breath transfer factor for carbon monoxide values for 11 CON and 12 MVV-infected sheep. *See* Figure 5 for key.

The 95.5% confidence interval for the difference in population medians for C_L ranged from −0.76 to 0.19 L·kPa^{-1} (Fig 3). This difference was not statistically significant.

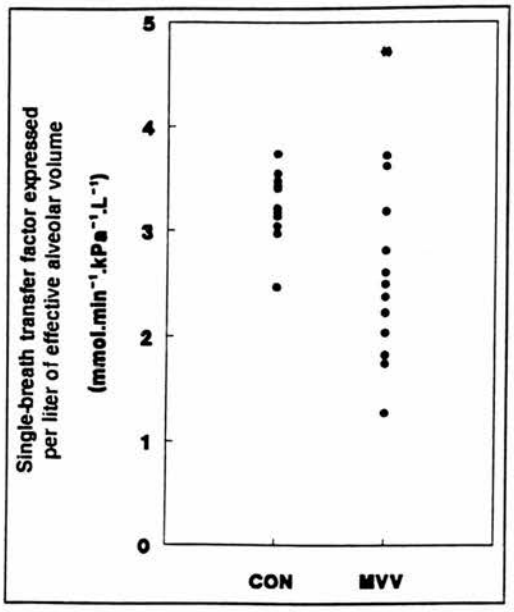

Figure 9—Transfer factor values expressed per liter of effective alveolar volume for 11 CON and 12 MVV-infected sheep. * Significantly ($P < 0.05$) lower than values for controls.

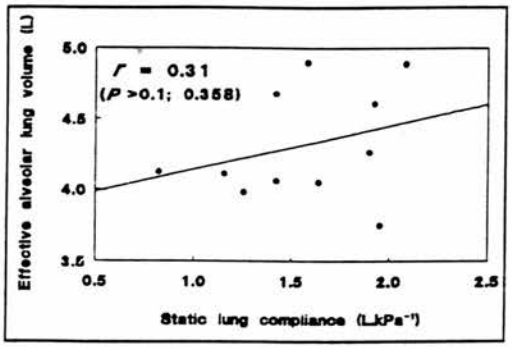

Figure 10—Relation between effective alveolar volume and static lung compliance for control sheep. The solid line represents the line of best fit by least-squares linear regression.

The 95.5% confidence interval for the difference in population medians for $V_{A\ eff}$ ranged from −1.53 to −0.43 L (Fig 4). This difference was statistically significant ($P < 0.01$).

The 95.5% confidence interval for the difference in population medians for $V_{EEL\ eff}$) ranged from −1.09 to −0.40 L (Fig 5). This difference was statistically significant ($P < 0.001$). Specific lung volume values were determined for individual sheep (Fig 6 and 7). Differences between groups were not statistically significant for $sV_{A\ eff}$ and $sV_{EEL\ eff}$.

The 95.5% confidence interval for the difference in population medians for transfer factor values ranged from −8.44 to −3.28 mmol·min^{-1}·kPa^{-1} (Fig 8). This difference was statistically significant ($P < 0.001$).

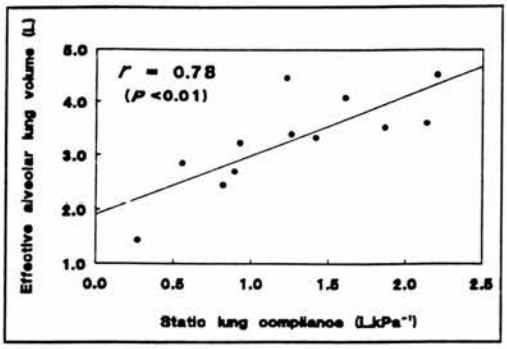

Figure 11—Relation between effective alveolar volume and static lung compliance for MVV-infected sheep. *See* Figure 10 for key.

Transfer factor values were also expressed per unit of alveolar volume (Fig 9). The 95.5% confidence interval for the difference in population medians for T_L/V_A ranged from −1.32 to −0.22 mmol·min^{-1}·kPa^{-1}·L^{-1}. This difference also was statistically significant ($P < 0.05$).

The relation between effective alveolar volume and C_L was determined for control (Fig 10) and MVV-infected (Fig 11) sheep. Results of least-square regression analysis relating C_L to body weight and $V_{A\ eff}$ to C_L were tabulated for control and MVV-infected sheep (Table 2).

Discussion

The history of the flock from which these MVV-infected sheep were derived has been described.[24] Postmortem examination of sheep from the parent flock have indicated that lungs with diffuse and extensive MVV lesions have a characteristic gross appearance.[24] On opening the thoracic cavity, the lungs tended not to collapse, appearing larger than normal. Lung weight was increased (0.52 to 1.96 kg; normal 0.4 to 0.6 kg), and the lungs were firm when cut and were rubbery. Consistent histopathologic features were smooth muscle hyperplasia and interstitial infiltration with lymphocytes, monocytes, macrophages, and some plasma cells.

Although all sheep of this study were adult, it is acknowledged that factors including age and time and route of infection would differ between individual sheep and, as such, they would probably have a spectrum of pulmonary pathologic features. Attempt was not made to grade or classify these sheep on a clinical basis; they represented a cross-section of the adult sheep within the flock.

The body weight and body condition score of MVV-infected sheep were significantly lower than values for the control sheep. With loss of body condition being a frequent early sign of MVV infection,[2,25] it is assumed that these observations are a feature of the disease process. Nutritional management of the 2 groups of sheep was identical and intercurrent disease was not apparent in either group on the basis of results of clinical or laboratory investigation. Weight

Table 2—Least-squares regression equations relating static lung compliance (C_L) to body weight (BW) and effective alveolar volume to C_L for control (CON) and MVV-infected sheep

Group	Dependent variable (unit)	Independent variable (unit)	Regression equation	F	r^2 (%)	P
CON	Static lung compliance ($L \cdot kPa^{-1}$)	Body weight (kg)	$0.414 + 0.0166 \times BW$	3.51	28.1	0.094
MVV	Static lung compliance ($L \cdot kPa^{-1}$)	Body weight (kg)	$-0.49 + 0.0318 \times BW$	2.44	19.6	0.149
CON	Effective alveolar volume (L)	Static lung compliance ($L \cdot kPa^{-1}$)	$3.83 + 0.310 \times C_L$	0.94	9.4	0.358
MVV	Effective alveolar volume (L)	Static lung compliance ($L \cdot kPa^{-1}$)	$1.89 + 1.11 \times C_L$	15.55	60.9	0.003

The coefficients of determination (r^2) in percentage units, F-ratios (F), and significance of the association between the variables (P) are shown.

loss and cachexia were commonly observed in the flock of origin.[24]

The normal pulmonary interstitium is composed of connective tissue components (collagen, elastic fibers, proteoglycans, and fibronectin), mesenchymal cells, and inflammatory and immune effector cells.[26] The most obvious physical effect of LIP is a thickening of the interstitial space, with consequent alteration of the number, form, and location of its cellular and noncellular elements. The anticipated physiologic consequences of such structural alterations include changes in compliance, lung volumes, and transfer factor. Because the pressure-volume characteristics of the lung are principally determined by the tissue network of collagen and elastic fibers (ie, the noncellular elements in the interstitium),[27] any change to this network is liable to lead to changes in compliance. Static lung volumes are influenced by lung and thoracic wall recoil pressures[23] and the volume of tissue and fluid within the thoracic cavity.[28] Changes in both determinants would be anticipated in LIP.

Defined as the rate of transfer of a gas per unit driving pressure between the alveoli and the erythrocytes within the pulmonary capillaries, transfer factor is affected by a number of structural lung dimensions, including lung volume, the path length for diffusion in the gas phase, the surface area of the alveolar capillary membrane, and the volume of blood in the capillaries perfusing ventilated alveoli.[22] Changes to several of these dimensions occur in LIP; thus, alterations in transfer factor would be anticipated.

The single-breath helium dilution method is commonly used to measure $V_{A\ eff}$ in human beings, though it does tend to underestimate the true lung volume in subjects with airflow restriction.[20] However, for subjects with only slight airflow limitation or interstitial lung disease, use of this technique is accepted.[29] The use of the derived measurement, $V_{EEL\ eff}$, is subject to the same limitations.

Use of the equation of Cotes[22] to correct transfer factor values for differences in hemoglobin concentration between individual sheep assumes that the reaction rates of carbon monoxide with sheep and human oxyhemoglobin are similar.

Fisher and Hyde[30] documented that pulmonary diffusing capacity and capillary blood flow were significantly reduced during passive lung inflation and breath-holding in anesthetized dogs. The cause of this reduction was attributed to decreased venous return to the right side of the heart and increased pulmonary vascular resistance. The magnitude of this reduction was significantly correlated with the level of transthoracic pressure used to induce lung inflation. In addition, a temporal change in diffusing capacity was detected, with a significant decrease in the first 7 seconds and no significant change between 7 and 11 seconds.[30] Therefore, it is necessary, when comparing single-breath transfer factor values between animals, to control lung inflation pressures and duration of breath-holding as was done in the sheep of this study.

The range of compliance values was wide for control and MVV-infected sheep. Halmagyi and Colebatch[17] studied anesthetized sheep (n = 27) with mean ± SD body weight of 42.6 ± 4.7 kg and reported dynamic lung compliance of 1.08 ± 0.316 $L \cdot kPa^{-1}$. By expressing this value per unit of body weight, an approximate value of 25 $ml \cdot kPa^{-1} \cdot kg^{-1}$ is obtained. This is in reasonable agreement with the value of 22.7 $ml \cdot kPa^{-1} \cdot kg^{-1}$ for our control sheep. In their series, Halmagyi and Colebatch obtained an intersubject coefficient of variation of 29% for measurements of dynamic compliance and commented that this degree of scatter was not dissimilar to that obtained in human beings. The coefficient of variation between control sheep for compliance measurements in this study was 25%; thus, intersubject variability was comparable to previous observations in anesthetized sheep. One factor that might contribute to this variation is body weight. Although compliance was positively correlated with body weight in control and MVV-infected sheep, the relation was not statistically significant as assessed by linear regression. Analysis of variance indicated that body weight accounted for only 19.6% of the variation in compliance in MVV-infected sheep and for 28.1% in the control sheep.

Given the observation that MVV-affected lungs tend not to collapse when removed from the thorax,[24] suggesting low compliance, lack of any significant difference between the groups was unexpected. Al-

though significant difference was not observed between the groups as a whole, the range of values of C_L was greater for the MVV-infected sheep with 5 individual sheep having $C_L < 1$ L·kPa^{-1}. Thus, changes in compliance may be a functional change only detectable at a particular stage of the disease, and perhaps if a more critical selection procedure had been used to select sheep (eg, on a clinical or age-related basis), significant differences would have been observed between MVV-infected and control sheep.

Changes in compliance in people with interstitial lung disease seem to reflect predominantly fibrosis[31] and are not correlated with inflammatory events.[32] In visna and maedi, the predominant lesions are cellular infiltration of the interstitium together with distinct smooth muscle hyperplasia. Although the exact pathophysiologic role of the smooth muscle hyperplasia is unknown, one theory is that it may serve to compensate for the lack of elastic recoil resulting from the thickening of the interalveolar septa and fragmentation and destruction of elastic fibers seen in the disease.[1] Accordingly, measurements of compliance in MVV-infected animals will presumably be influenced by the quantity and functional tone of the smooth muscle in the lungs.

Previously reported[33] static lung volume values—TLC of 76 ml·kg^{-1} and FRC of 25 ml·kg^{-1}—in unanesthetized sheep compare with values of 64 ml·kg^{-1} for $V_{A\ eff}$ and 31 ml·kg^{-1} for $V_{EEL\ eff}$ in our sheep. The differences in these measurements likely reflect the effects of anesthesia and different methods used in these studies. Intersubject coefficients of variation of 10 to 15% were reported[33] for static lung volume measurements and compare favorably with our coefficients of variation of 9% for $V_{A\ eff}$ and 14% for $V_{EEL\ eff}$.

Lung volumes were significantly lower in MVV-infected sheep than in controls; however, when corrected for body weight, significant difference between the groups could not be detected. It might be reasoned that the difference in lung volumes between the groups is simply a reflection of different body sizes, with a normal ratio of lung volume to body weight being maintained in the smaller MVV-infected sheep. However, our subjective opinion that the sheep of both groups had similar skeletal dimensions, coupled with the significantly poorer condition scores of MVV-infected sheep, counters this argument and suggests that there is a concomitant decreased in body weight and lung volume as a result of the disease process. To support this argument, that the smaller lung volumes of the diseased sheep are a reflection of the disease process, is the observation that compliance shares a significant linear relation with lung volume in MVV-infected sheep ($r = 0.78$; $P < 0.01$), whereas no such relation can be documented for the control sheep ($r = 0.31$; $P > 0.1$). Indeed, compliance accounted for 60.9% of the variation in lung volume in MVV-infected sheep and only accounted for 9.4% in the control sheep. In contrast, body weight accounted for 33.3% of the variation in lung volume in the control sheep and for only 11.3% in MVV-infected sheep. Thus, a major influence on lung volume in MVV-infected sheep would appear to be compliance.

Similar changes in lung volumes are recognized in human beings with idiopathic pulmonary fibrosis, in whom volume reduction may also be accompanied by a decrease in lung compliance.[34] A reduction in lung volume is also a common feature of sarcoidosis[35] and may be seen in people with LIP.[10,36]

Transfer factor per se and transfer factor corrected for alveolar lung volume values were significantly reduced in MVV-infected sheep. This is a common finding in human beings with interstitial lung disease[26] in whom transfer factor measurements may be useful for the follow-up evaluation of individual cases of sarcoidosis and interstitial fibrosis.[37] The $V_{A\ eff}$ value is the single greatest source of variability of $T_{L\ CO\ sb}$[29] with a decrease in $V_{A\ eff}$ exposing a smaller alveolar membrane surface area to participate in gas exchange. The significantly lower $V_{A\ eff}$ in MVV-infected sheep may, therefore, contribute to the observed difference in $T_{L\ CO\ sb}$. That other factors are involved in the reduction of the transfer factor in MVV-infected sheep is evidenced by the still significant reduction in transfer factor after correction for lung volume. Such factors are likely to include alterations in ventilation-to-perfusion ratio, pulmonary hypoperfusion, capillary transit time, and thickening and functional alterations of the interalveolar membrane brought about by the cellular infiltration.[38]

In people with interstitial lung disease, ventilation-perfusion inequalities account for the major part of observed hypoxemia.[34] These inequalities, which are thought to arise as a result of nonuniform alterations to the mechanical properties of the lung parenchyma,[39] can reduce the effective interface for gas transfer and complicate the interpretation of transfer factor measurements made using steady-state methods.[23] However, for the single-breath technique, where a uniform distribution of the inspirate can be anticipated, maldistribution of ventilation to perfusion is unimportant unless it is extreme.[40] Other unequal distributions (eg, the distributions of diffusion properties relative to alveolar volumes and pulmonary capillary blood flow throughout the lung), are likely present in MVV-infected sheep, and may reduce apparent single-breath transfer factor values with increasing time of breath-holding.[40] Despite these latter limitations, single-breath transfer factor values should provide a useful numerical index of the overall transfer characteristics of the lungs in MVV-infected animals.

These physiologic changes, specifically reduction in lung volumes and transfer factor, are consistent with those found in people with interstitial lung disease.[7,8] Knowledge that measurable changes in lung function do occur in natural MVV infection provides a basis for further investigation of the potential of lung function studies as a means of staging this disease.

Sheep are useful experimental animals in pulmonary research—their lungs being of comparable size to those of human beings.[41] In addition, sequential analysis of the bronchoalveolar milieu of con-

scious sheep by physiologic, histologic, and bronchoalveolar measurements is well tolerated, with reproducibility and variations of measurements being similar to those found in human beings.[33] Accordingly, integration of these techniques with recently developed immunologic and molecular biological technologies will provide a powerful means for investigating the pathogenesis of LIP induced by ovine lentivirus infection.

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Exponential analysis of the pressure-volume characteristics of ovine lungs

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Abstract. Static pressure-volume curves were generated from data obtained from 18 normal anaesthetized adult sheep. Lung volumes were determined by helium dilution. An exponential curve of the form $V = Vmax - Ae^{-KP}$ was fitted to the pressure-volume data from each sheep where P is the static recoil pressure. Vmax represents the volume asymptote. A is the difference between Vmax and the intercept on the volume axis and K defines the slope and hence the shape of the P-V curve. Quality of fit of the data was assessed visually, by means of a sign test and a runs test and by the coefficient of determination (r^2). Exponential equations were found to adequately describe the shape of the pressure-volume curve in sheep. The exponent K was not correlated with effective alveolar volume (VA_{eff}) ($r_s = 0.183$; $P > 0.05$). Static lung compliance was determined over a volume range from the end-expiratory level (VEEL) to VEEL plus 400 ml. Measurements of static lung compliance were significantly correlated with measurements of effective alveolar volume (VA_{eff}) ($r_s = 0.505$; $P < 0.025$). In the ovine, the exponent K, an index of distensibility, is independent of lung volume and offers a means of assessing lung distensibility in this species.

Distensibility, lung: Mammals, sheep: Mechanics of breathing, static pressure-volume: Pressure, static, lung volume: Volume, lung, static pressure

Static lung compliance (Cst), analysed as the lung volume change per unit pressure change (dV/dP) in the tidal volume range, is often assumed to be an index of lung elastic recoil, and is frequently used as such. Unfortunately, when comparing animals of different body size or comparing animals with varying stages of lung disease, the above assumption is subject to qualifications which limit the value of this measurement.

Firstly, variation in the end-expiratory level will alter the portion of the curve over which the compliance measurement is made *i.e.* the measurement of compliance will depend on the end-expiratory lung volume, and secondly, compliance values intuitively depend on the inflatable volume of the lungs themselves. Thus, accurate interpretation

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of compliance values demands both a knowledge of the lung volumes over which the measurement is made and a perception of the predicted normal value for the animal concerned. These latter requirements place an extra burden on measurement protocol and rely heavily on the accuracy of prediction equations based on anthropometric data.

An alternative means of analysing pressure-volume curves was pioneered by Salazar and Knowles (1964) who used an exponential equation to describe the pressure volume curve of human adults. Glaister *et al.* (1973) employed the same principle to describe the pressure volume curves of excised dog and monkey lungs. The basis behind this method of analysis, which utilizes all the pressure and volume data points collected, has been developed and applied to the analysis of pressure-volume curves from both healthy adults (Pengelly, 1977; Colebatch *et al.*, 1979a,b; Gibson *et al.*, 1979; Knudson and Kaltenborn, 1981; Colebatch and Ng, 1986) and those with respiratory disease (Gibson *et al.*, 1979; Colebatch *et al.*, 1985; Thompson and Colebatch, 1989). The exponent K of the fitted exponential equation is not influenced by absolute values of pressure or volume and only delineates the relative changes of one with respect to the other. In other words, K is an index of pulmonary distensibility and appears not to suffer from the aforementioned limitations of compliance measurements.

Due to structural and functional similarities between the lungs of sheep and those of humans, this species has frequently been used to model human lung disease. Although pulmonary mechanics measurements are frequently included in such studies, there has, to the authors knowledge, been only one attempt to fit an exponential equation to the normal ovine pressure-volume curve (Schroter, 1980). In view of the relative advantages of expressing pulmonary distensibility in this way we sought to determine whether the ovine pressure-volume curve could be adequately described by an exponential equation and to report normal values of K for this species such that in future the distensibility of the ovine lung could be accurately assessed in disease and in relation to other species.

Materials and methods

Animals. 18 adult Texel and Texel-cross female sheep (median bodyweight 63.5; range 54–82 kg) were used in this study. The sheep were fed on a diet of proprietary concentrate and hay and were assessed to be free of significant cardiopulmonary dysfunction on the basis of a thorough clinical examination.

Anaesthesia. Sheep were starved for 12 h prior to anaesthesia which was achieved using intravenous administration of thiopentone sodium at a dose rate of 20 $mg \cdot kg^{-1}$ bodyweight. The sheep were then intubated using cuffed endotracheal tubes (diameter 9.5–10.5 mm) and placed in sternal recumbency with the head supported on a cushioned rest. A mechanical ventilator (Manley MN2; Hutchinson Blease) was adjusted to maintain a tidal volume of 10 $ml \cdot kg^{-1}$ bodyweight and respiratory rate of 10 $breaths \cdot min^{-1}$.

Measurements. Transpulmonary pressure was measured using a differential pressure transducer (CS9; Mercury Electronics) which was connected to an oesophageal balloon catheter assembly and to a port at the proximal end of the endotracheal tube. The catheter (3 mm ID, 4.5 mm OD) had a number of holes cut in a spiral manner in the end covered by a latex balloon (length, 15 cm; diameter, 1.5 cm; thickness, 0.05 mm) and this assembly was passed into the middle thoracic portion of the oesophagus. Prior to each series of measurements, the pressure-volume characteristics of the balloon-catheter assembly were validated according to the method of Senterre and Geubelle (1970). The balloon was then evacuated and 2 ml of air was added (this volume being within the range of high compliance of this pressure recording system and close to the minimal relaxed volume of the balloon in air). The positioning of the balloon catheter assembly was confirmed by observing cardiogenic oscillations in the transpulmonary pressure recorder trace and by the absence of any deflection of this trace during inspiratory efforts against a closed airway (Milic-Emili *et al.*, 1964). Respiratory flows were measured using a Fleisch pneumotachograph (No 2) connected to a second differential pressure transducer (CS9; Mercury Electronics). Integration of the derived signal yielded respiratory volumes which were displayed on the chart recorder (Linseis L6514; Belmont Instruments). Pressure and volume recording equipment were calibrated using a water manometer and calibrated syringe respectively.

Lung volumes were measured using a single-breath helium dilution technique performed in conjunction with the measurement of single-breath transfer factor as previously described (Collie *et al.*, 1993). Lungs were inflated from the resting end expiratory level to a transpulmonary pressure of 3 kPa (defined as total lung capacity (TLC)) with a carbon monoxide and helium in air gas mixture (helium 14%, carbon monoxide 3%, balance air). After a 10-sec breathhold, a sample of alveolar gas was obtained and the concentration of helium determined (Resparameter Mk 4; P.K. Morgan). Effective alveolar lung volume (VA_{eff}) was calculated as previously described (Collie *et al.*, 1993).

Pressure-volume data were obtained after three forced inflations to TLC. The lungs were again inflated to TLC, however a stepwise deflation was achieved by intermittently interrupting the airway opening for 2–3 sec intervals.

Three collections of lung volume and pressure-volume data were made on each animal during the period of anaesthesia. To obtain absolute values for pressure-volume curves the expired volumes were subtracted from the VA_{eff} values determined immediately prior to collection of pressure-volume data. Static lung compliance (Cst) was calculated as the slope of the pressure-volume curve between the end-expiratory level and this level plus 400 ml.

Exponential analysis of pressure-volume curves. An exponential curve of the form $V = Vmax - Ae^{-KP}$ was fitted to the data from each sheep where P is the static recoil pressure, Vmax represents the volume asymptote, A is the difference between Vmax and the intercept on the volume axis and K represents the slope of the P-V curve and therefore defines its shape. The method of Pengelly (1977) was used to determine the

exponential function that best fitted the data. Briefly, by expressing the difference between Vmax and the measured volume as a fraction of Vmax and taking the natural logarithm of this value a straight line plot with P is obtained. Least squares regression is then used to solve for slope and intercept. However, since Vmax is unknown at the outset an iterative regression is conducted until values of the coefficient of determination (r^2) are maximized. Curves were fitted both over the whole data range and over a restricted data range in which the data points less than 50% of TLC were excluded from the analysis. In addition to assessing r^2, curves were evaluated visually for goodness of fit and both a sign test for distribution of the data about the fitted regression line and a runs test to determine whether the order of the data was random ($P < 0.05$) were performed (Gibson *et al.*, 1979). Associations between variables were assessed using the Spearman rank correlation coefficient (r_s)

Results

The summary results of curve-fitting analysis over the whole data range for 18 sheep are shown in Table 1. The pressure-volume data from two sheep with exponential curves fitted are presented in Fig. 1. These examples represent the opposite ends of the spectrum in terms of goodness of fit as assessed by r^2. In general the regression lines appeared to closely fit the pressure-volume data on visual inspection. Although only one pressure-volume curve failed the sign test ($P < 0.05$), all but two failed the runs test ($P < 0.05$) indicating that there were systematic deviations from the fitted regression lines in almost all the sheep *i.e.* the order of these deviations was not random. The nature of these deviations is demonstrated in a plot of the residuals against pressure (Fig. 2) (with pressures expressed as percentages of the range between the minimum (0%) and maximum (100%) pressure recorded for each set of data points). The most obvious systematic deviations from the fitted regression lines occurred when transpulmonary pressure was maximal with measured volumes being greater than the

TABLE 1

Summary results from exponential pressure-volume curve fitting analysis of the form $V = V_{max} - Ae^{-KP}$ in 18 normal sheep. V_{max} is the volume asymptote. A is the difference between V_{max} and the intercept on the volume axis. K defines the slope and hence the shape of the fitted pressure-volume curve and r^2 is the coeffcient of determination of the regression. Cst is the static lung complicance.

Measurement	Median	Range
Data points/animal	41	26–48
K (kPa^{-1})	1.456	1.091–2.119
A	2542	1590–3252
V_{max} (ml)	4168	3277–4896
r^2	98.74	96.11–99.74
Cst ($l.kPa^{-1}$)	2.050	1.131–3.698

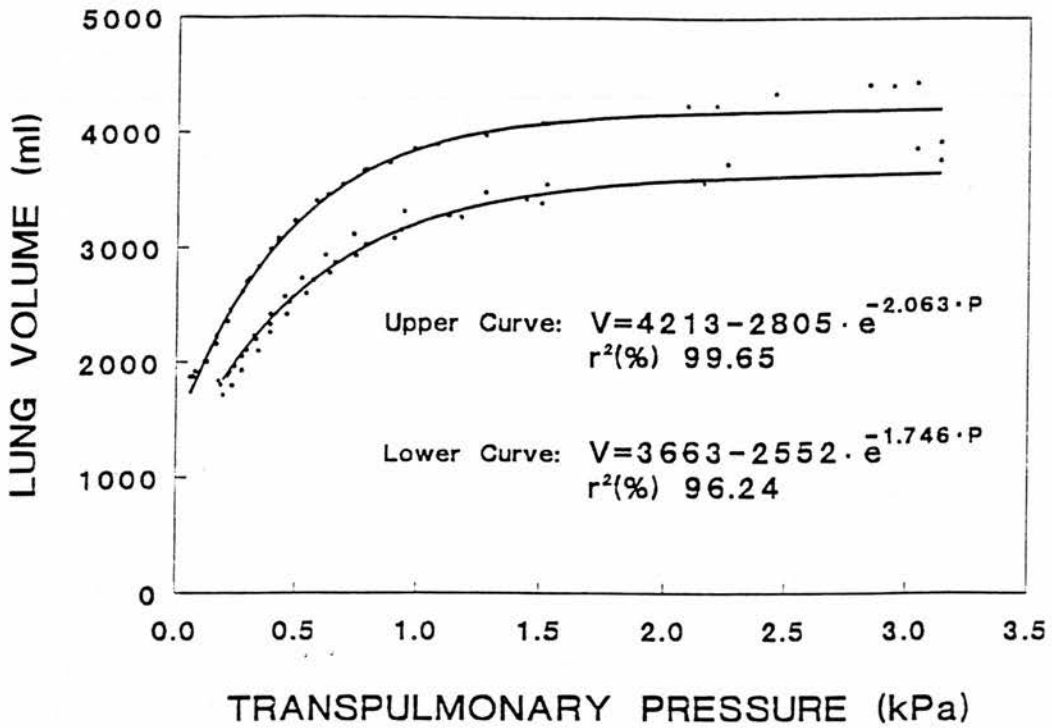

Fig. 1. Pressure-volume curves from two normal sheep. The two curves are representative of the extremes of quality of fit of data.

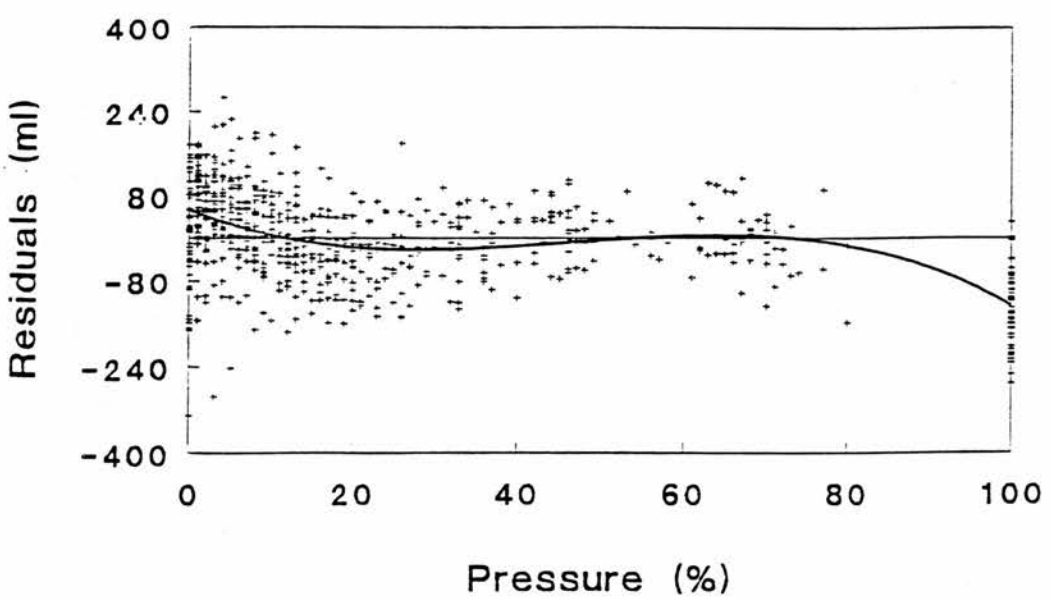

Fig. 2. Plot of residuals for all fitted curves for 18 sheep. Pressures are expressed as percentages of the range between the minimum (0%) and maximum (100%) pressure recorded for each set of data points. The fitted curve is of the form $Y = a + b \cdot x + c \cdot x^2 + d \cdot x^3$.

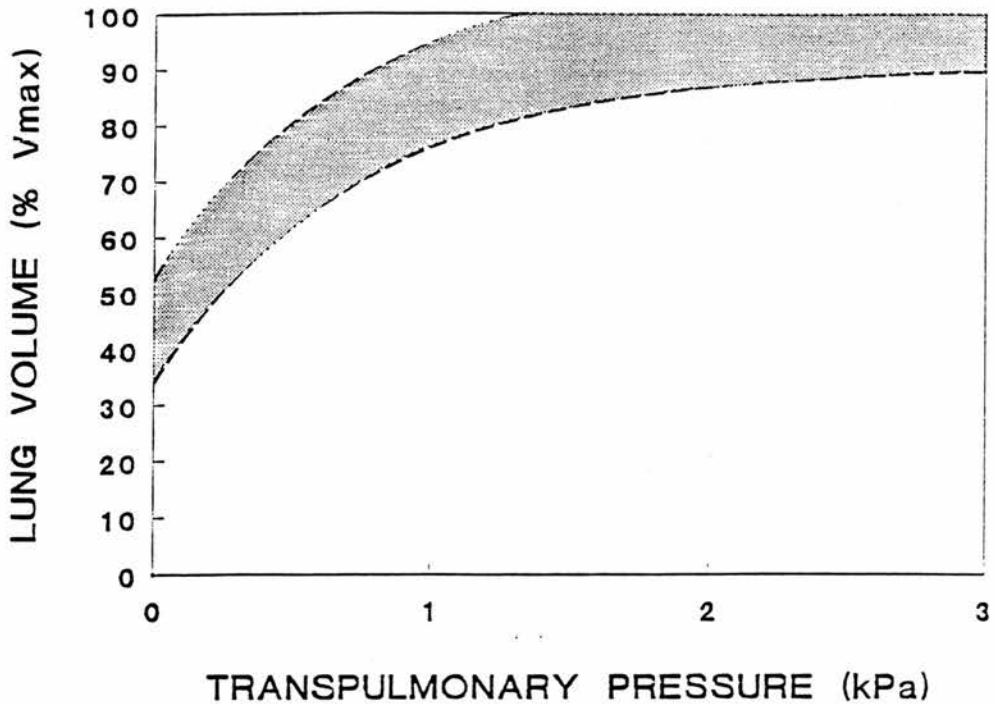

Fig. 3. Relationship between transpulmonary pressure and lung volume (expressed as percentage of predicted Vmax) in normal sheep. The shaded area represents the 95% confidence interval.

regression would seem to predict. Curves fitted data well at lower volume limits. Curve fitting over the restricted data range improved the quality of fit (as assessed by r^2) in only one instance. In the remaining sheep, fitting a curve to the restricted data range led to a slight reduction in r^2 (median 98.34;range 95.42–99.39) and K (median 1.348;range 0.820–2.098), and an increase in Vmax (median 4202;range 3468–4896).

The iterative regression was repeated on the pooled data from all sheep with volume expressed as percent of Vmax. The exponential equation $V/Vmax\ (\%) = 100 - 56.7 \cdot e^{-1.37 \cdot P}$ was found to best fit this pooled data. The relationship between transpulmonary pressure and lung volume expressed as percent predicted Vmax is depicted in Fig. 3 with the shaded area representing the 95% confidence interval for normal sheep.

A significant positive correlation was apparent between Cst and VA_{eff} ($r_s = 0.505$; $P < 0.025$) whereas no significant relationship existed between K and VA_{eff} ($r_s = 0.183$; $P > 0.05$).

Discussion

The best-fit exponential curve was chosen by maximizing the coefficient of determination obtained by least-squares regression analysis of the linearised data. Visually, exponential curves appeared to closely fit the pressure-volume data and this was reflected

in the regression analyses, where 96.11–99.74% of the variation in the dependent variable was explained by the fitted curve. Indeed, in only 4 cases did the unexplained variation exceed 2% of the total variation.

Systematic deviations from the exponential functions were frequently noted at high transpulmonary pressures and much of the error variance was attributable to this portion of the curve. The implications of these observations are that (a) in sheep, lung volumes measured at a transpulmonary pressure of 3 kPa are not maximal, and (b) an exponential curve is not the most accurate description of the relationship between transpulmonary pressure and lung volume in this species. Examination of a previously published pressure-volume curve obtained from an anaesthetized sheep (Colebatch and Halmagyi, 1961) suggests that the first assumption might be valid. With regard to the second assumption, it is quite likely that a more complex mathematical formulation could be derived to better fit the pressure-volume data from sheep, just as in humans a hyperbolic-sigmoid model appears to offer a better fit to the full range of pressure-volume data than the exponential model (Murphy and Engel, 1978). However, the relative simplicity and lack of constants in the exponential equation together with the predictable effect of a change in any constant on the shape and position of the curve weigh heavily in its favour and, in our opinion, compensate for the poor description of the curve at high transpulmonary pressure. Certainly, it would appear that the exponential constant K holds several advantages over Cst measurements in describing pressure-volume curves. Firstly, the full range of data is used in the exponential analysis, whereas only data points in the linear portion of the pressure-volume curve are used in the calculation of Cst. Secondly, whereas Cst is positively correlated with VA_{eff}, K is independent of this variable and thus is a more useful index of distensibility for use in comparative studies or when studying the effects of lung disease on pulmonary mechanics.

Data points at low volume levels tend not to conform to the exponential model in humans (Colebatch *et al.*, 1979a; Gibson *et al.*, 1979). Below about 46% TLC in seated healthy men (Sutherland *et al.*, 1968) there is an inflection in the curve caused primarily by the sequential closure of small airways and oesophageal artefacts. Inclusion of data points below this point of airway closure in the exponential curve fitting analysis leads to underestimation of K and overestimation of Vmax (Gibson *et al.*, 1979). Inflection points were not well defined in our ovine pressure-volume curves and curves appeared to underestimate Vmax rather than the converse. In addition, exclusion of data points below 50% TLC did not improve the quality of curve fit. We therefore conclude that the factors responsible for curve inflection have less influence in the low volume range in anaesthetized sheep relative to humans and the whole data range from VEEL to TLC can be used for exponential curve fitting.

The results of the pooled regression indicate that the ratio of A/Vmax% is approximately 57%. This ratio describes the position of the curve relative to the transpulmonary pressure axis and is considerably lower than the equivalent ratio previously described for humans (Colebatch *et al.*, 1979a; Gibson *et al.* 1979; Knudson and Kaltenborn, 1981) indicating that relative to these studies, the sheep pressure-volume

curves are displaced to the left. Knudson and Kaltenborn (1981) reason that differences in oesophageal balloon volume can account for much of the variation in this ratio seen in human studies and comment that, compared to measurements of K which are seemingly less variable between studies, the value of this ratio to compare various studies is limited.

K does not seem to be volume dependent in humans (Colebatch *et al.*, 1979a) although a progressive increase in this parameter occurs with age (Colebatch *et al.*, 1979b) such that between 20 and 80 years of age the value of K would be expected to increase from approximately 1.224–1.748 kPa. The age related increase in K is believed to be due to increased unstressed alveolar dimensions and reduced total surface acting forces (Colebatch and Ng, 1986). This relationship between airspace size and K has been demonstrated in excised human lungs (Greaves and Colebatch, 1980) and for lungs of several mammalian species (Haber *et al.*, 1983). Thus, species with very small alveoli would be expected to have relatively large surface acting forces and low values of K. In this regard it is interesting that the values of K we have obtained for adult sheep (median 1.456 kPa; range 1.091–2.119) are similar to those reported for humans; this despite sheep having smaller alveolar dimensions and correspondingly greater alveolar surface density relative to humans (Gehr *et al.*, 1978; Warner *et al.*, 1986). Whether this represents an intrinsic difference between species with regard to the type and extent of tissue related forces or a difference due to methodological inconsistencies between studies remains to be ascertained.

In conclusion, our studies indicate that an exponential equation adequately describes the whole pressure-volume curve of normal anaesthetized sheep. The index of distensibility K, is independent of lung volume and offers a means of comparing lung distensibility both with other species and during lung disease in this species.

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Pathophysiologic Correlations in Lymphoid Interstitial Pneumonia

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Effective alveolar volume, diffusing capacity for carbon monoxide (DCO_{sb}), volume-corrected diffusing capacity (D/VA), static lung compliance (Cst), and lung distensibility were measured in 16 sheep seropositive for maedi-visna virus (MVV) immediately before they were killed. Lungs were inflation-fixed, and the left lung was randomly sampled for morphometric analysis. The total lung weight, total fixed lung volume, volume densities of tissue (Vvt) and air (Vva), and the alveolar surface density were measured and correlated with the physiologic measurements. The density of surface forces could not account for the variation in the distensibility of the lungs, indicating that tissue-related forces may be important in determining lung distensibility in lymphoid interstitial pneumonia (LIP) associated with MVV infection. Possible sources of tissue-related forces are the contractile tissue associated with lung parenchyma, airways, or vasculature. When DCO_{sb} was corrected for volume, a strong negative correlation with Vvt was noted, indicating that factors distinct from lung-volume reduction are important in limiting gas exchange in LIP associated with MVV infection. More sheep demonstrated abnormal D/VA values than any other physiologic measurement, with reduced values being apparent even in sheep considered clinically normal and with little or no morphometric evidence of lung disease. Measurements of diffusing capacity are thus considered the most sensitive functional index of disease progression. **Collie DDS, Warren PM, Begara I, Luján L, Watt NJ. Pathophysiologic correlations in lymphoid interstitial pneumonia. Am J Respir Crit Care Med 1994; 149:1575–82.**

Lymphoid interstitial pneumonia (LIP) is characterized by the interstitial accumulation of lymphocytes, macrophages, and plasma cells. In humans, this condition may accompany several immunologically mediated diseases, and it also occurs in association with HIV infection, particularly in children (1). No clinical feature or noninvasive test can specifically confirm the presence of LIP, and lung biopsy is the only means of establishing a definitive diagnosis of this condition (2).

Physiologically, the human interstitial lung diseases are generally characterized by a reduction in lung volumes, lung compliance, and diffusing capacity (3). Serial measurements of lung function indices are used in the assessment and management of LIP (4). However, attempts at relating functional abnormalities to histologic indices of disease activity in interstitial lung disease are often disappointing (5, 6) with functional indices roughly reflecting the overall pathology but being relatively insensitive. This insensitivity may be due in part to both sampling problems, i.e., extrapolating morphologic data from a biopsy specimen to the whole lung (5), and to temporal separation of the functional tests from necropsy examination of the lung (7).

If animal models of human disease are used, however, then an accurate qualification and quantification of pulmonary disease can be made at necropsy examination. Correlation of these data with functional measurements made immediately before the animal is killed allows a more accurate assessment of the specificity of such measurements to be made. In sheep, natural and experimental infection with the ovine lentivirus, maedi-visna virus (MVV), causes a chronic progressive lymphoproliferative disease that affects the lungs, nervous system, mammary glands, and joints (8, 9). The pathologic features of this respiratory disease are essentially identical to those described for LIP associated with HIV infection in humans (9), and MVV shares morphologic and sequence homology with HIV (10). Thus, an appropriate animal model for LIP, particularly that associated with HIV infection, exists in sheep, an animal with lungs of comparable size and similar structure to those of human beings and in which the lung functional abnormalities associated with MVV infection have recently been characterized (11).

Knowledge of how functional measurements in LIP associated with MVV infection specifically relate to structural indices of disease severity will be of value in staging and managing the disease in sheep, and it will be particularly relevant to the assessment of therapeutic strategies in this condition, an area of immediate significance to the management of LIP associated with HIV infection. This report describes the objective quantitative assessment of LIP associated with MVV infection and presents the

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relationships that exist between functional and structural indices in this condition.

METHODS

Animals

Sixteen adult Texel ewes (median body weight, 57.5 kg; range, 49 to 67 kg), seropositive for MVV on the basis of results of the agar gel immunodiffusion test (12), were used in this study. These sheep were selected from a flock, the history of which has previously been described (13). Sheep were housed during the period of physiologic study. Neither clinical nor routine hematologic examinations indicated the presence of significant respiratory disease unrelated to infection with MVV. Subsequent necropsy examination confirmed the absence of significant respiratory disease unrelated to MVV infection in these sheep.

Clinical Studies

A subjective clinical grading of severity of respiratory disease was made on each sheep. A grade of zero was assigned when there was no clinical evidence of respiratory disease, a grade of 1 was assigned when the only evidence of respiratory disease was an increase in adventitious sounds on auscultation, and a grade of 2 was assigned when there was, in addition, evidence of dyspnea with or without mouth breathing. Body condition scores were assessed on a scale of zero to 5, with zero representing extreme emaciation and 5 representing excessive fat cover (14).

Physiologic Studies

Functional studies were executed as previously described (11, 15). Briefly, measurements were conducted on anaesthetized sheep in sternal recumbency with the head supported on a cushioned rest. Mechanical ventilation was maintained throughout the period of measurement. Transpulmonary pressure (P_L) was measured using a pressure transducer (CS9; Mercury Electronics, Glasgow, UK) connected to an esophageal balloon catheter assembly and a similar catheter connected to a sidearm proximal to the endotracheal tube. Respiratory flows were measured using a pneumotachograph (Fleisch No. 2) and transducer system (CS9; Mercury Electronics) connected to the proximal end of the endotracheal tube. The resulting signal was integrated to yield respiratory flows. Pressure-measuring devices were calibrated using a water manometer, and volume-measuring devices were calibrated using a calibrated syringe.

Static lung volume, i.e., effective alveolar volume (V_{Aeff}) was measured using a helium dilution technique performed in conjunction with the measurement of single-breath diffusing capacity for carbon monoxide ($D_{CO_{sb}}$). Blood hemoglobin concentration was measured, and values of $D_{CO_{sb}}$ were adjusted to a standard hemoglobin concentration using the equation of Cotes (16). Three determinations of lung volume and diffusing capacity were made on each sheep, and the mean value was calculated.

Pressure-volume data were obtained after three forced inflations to TLC. The lungs were again inflated to TLC, and a stepwise deflation was achieved by intermittently interrupting the airway opening for 2- to 3-s intervals. Three stepwise deflations were completed, and absolute-volume values were obtained by subtracting the expired volumes from the V_{Aeff} values determined immediately prior to collection of pressure-volume data. An average of 41 data points (range, 20 to 56) were collected from each animal. Static lung compliance (Cst) was measured on the expiratory limb of static pressure-volume curves between FRC and 40% of the way from FRC to TLC, i.e., the lung volume at a P_L of 3 kPa. The mean of three Cst determinations was calculated.

An exponential curve of the form $V = Vmax - Ae^{-KP}$ was fitted to the data from each sheep where P is the static recoil pressure, Vmax represents the volume asymptote, A is the difference between Vmax and the intercept on the volume axis, and K represents the slope and hence defines the shape of the P-V curve. The method of Pengelly (17) was used to determine the exponential function that best fitted the data. K is an index of pulmonary distensibility independent of lung size (Collie and coworkers, 1993; unpublished data).

Tissue Preparation

After the physiologic measurements the sheep were killed by intravenous injection of barbiturate and were exsanguinated. The lungs were carefully removed from the thorax, and the heart, great vessels, and caudal mediastinal lymph nodes were dissected free. Gross pathologic features were recorded. Both the total lung weight and the left lung weights were recorded. After clamping the lobar bronchi to the right lung, the trachea was cannulated and connected to a reservoir of phosphate-buffered 4% paraformaldehyde (pH, 7.2 to 7.4), at a head pressure of 2.5 to 3.0 kPa. This fixative was allowed to flow until the "natural contours" of the lung were established. The left lungs were then floated in a tank of the same fixative and inflation-fixed at a pressure of 2.5 to 3.0 kPa for a period of 4 d. Random tissue samples were taken as described in the morphometric analyses section. Tissue blocks were embedded in paraffin, and sections were cut at 7 μm and stained with hematoxylin-eosin.

Morphometric Analyses

The volume of the left lung was determined by water displacement (18), and the total lung volume (TLV_t) was inferred from the ratio of left lung weight to total lung weight. The principle of stratified random sampling was applied to selection of tissue blocks for subsequent analysis. The lung was sectioned into 1-cm-thick transverse slices, and six slices from the diaphragmatic lobe were selected for tissue sampling. Using a transparent numbered grid overlay (19) and computer-generated random numbers, a total of 12 tissue blocks were selected from the six slices. Tissue blocks were not accepted if the selected area overlay large conducting airways or blood vessels. Tissue blocks were trimmed to 1.9 × 1.9 cm using a suitable template. Morphometric analyses were performed by light microscopy at a magnification ×400 using a multipurpose test grid (Weibel GW2; Graticules Ltd., Graticules, Ltd., Tonbridge, Kent, UK) composed of a combined line and point system (20). All morphometric analyses were completed by the same operator. The volume densities of tissue (Vvt) and air (Vva) were calculated by relating the number of points landing on tissue to the total number of points counted, and the surface density (Svt) was calculated by relating the number of line intersections with alveolar septae to the number of tissue point "hits" (21). Tissue sections were randomly selected from the pool of 12 sections for each sheep, and six fields from each section were randomly selected and analyzed as described above (after analysis each section was returned to the pool and could be randomly selected again). A total of 72 fields were therefore examined, giving a total point count of 3,024 points per animal. Only parenchymal fields were analyzed, with parenchymal fields being defined as fields with no airways or blood vessels greater than 1 mm in diameter present. Values were multiplied by the total volume of the parenchymal region to calcu-

TABLE 1

CLINICAL AND BODY WEIGHT DATA FROM THE SHEEP IN THE PRESENT STUDY

Sheep No.	Body Weight (kg)	Age (yr)	Condition Score	Clinical Score
07	49	5.4	1.0	0
12	67	> 6.9*	1.5	0
17	50	5.2	1.5	1
32	55	6.7	2.0	0
53	50	5.9	2.0	2
59	52	> 7.3*	1.0	1
68	52	6.0	1.0	0
73	50	6.5	1.0	0
74	60	6.0	1.0	2
79	51	5.4	1.5	2
89	65	> 6.9*	2.5	1
90	67	5.9	3.0	0
92	64	5.9	2.0	0
93	62	> 6.9*	2.5	0
96	62	5.9	2.5	1
97	60	5.9	2.5	0

* Actual birth date unknown.

late absolute volumes and surface areas. The parenchymal fraction of the total volume was estimated as 0.85, based on point counting of lung slices (Collie and coworkers, 1993; unpublished data). Because all lungs were treated in an identical manner, it was considered unnecessary to calculate fixation or processing constants.

Statistical Analysis

To facilitate discussion of the analysis, data were grouped as follows: (*1*) clinical data, including age, bodyweight (BW), body condition scores, and subjective grading of disease severity; (*2*) physiologic data, including Cst, K, VAeff, DCO_{sb}, and DCO_{sb} values corrected for alveolar volume (D/VA); and (*3*) morphometric data, including total lung weight (TLW), TLV_t, Vvt, Svt, and the absolute parameters total alveolar volume (TVA) and total air-space surface area (ASA) derived from these data. Spearman's rank correlation coefficient (r_s) was used to compare within- and between-group values, and $p < 0.01$ was accepted as an appropriate level of significance. Where a large proportion of tied ranks were present in the data, a correction factor was used to correct the sum of squares (22). Physiologic measurements were also expressed as percent predicted values, these values being obtained from previously generated regression equations (15). Linear regression analysis was used when appropriate.

RESULTS

Clinical Data

Clinical data are shown in Table 1. Nine sheep had no clinical evidence of respiratory disease (Grade 0), four demonstrated an increase in adventitious sounds on auscultation (Grade 1), and three had, in addition, more overt signs of respiratory disease (Grade 2). BW was positively correlated with condition score (r_s, 0.633; $p < 0.01$); however, neither BW nor condition score was correlated with clinical score.

Morphometric Data

Mean values for morphometric data are shown in Table 2. Lung weights ranged from 0.55 to 1.74 kg. Vvt ranged from 17.0 to 50.2%, and absolute estimates of TVA ranged from 1,025 to 2,198 ml. Surface density of the alveolar epithelium varied from 243.4 to 684.9 cm^2/cm^3, and total gas exchange surface area varied from 48.7 to 156.7 m^2.

Physiologic Data

Physiologic data are illustrated in Figure 1. Values are expressed as percentage of predicted values for sheep of the same body weight and breed (15). The majority of sheep ($n = 12$) had Cst values in the normal range (Figure 1A). Seven sheep had VAeff values in the normal range (Figure 1B). Although four sheep had DCO_{sb} values within the normal range (Figure 1B), only two of these sheep had D/VA values in the normal range (Figure 1C). Values of K ranged from 0.685 to 1.866 kPa (normal range, 1.091 to 2.119 kPa; Collie and coworkers, 1993; unpublished data), and the median coefficient of determination (r^2, expressed as a percentage) was 99.16 (range, 95.27 to 99.83) (Figure 2).

Comparison of Clinical Data with Morphometric and Physiologic Data

Surface density was positively correlated with BW (r_s, 0.662; $p < 0.005$) and body condition score (r_s, 0.590; $p < 0.01$). Condition score was negatively correlated with Vvt (r_s, −0.590; $p < 0.01$). Clinical score was positively correlated with TLW (r_s, 0.615; $p < 0.01$). There were no significant correlations between clinical score, condition score, and BW and the physiologic data.

Comparisons between Morphometric Parameters

Notable correlations were the strong negative correlation between TLW and Svt (r_s, −0.693; $p < 0.0025$) and the strong positive correlation between TVA and total gas exchange surface area (r_s, 0.826; $p < 0.0005$). TLV_t was not significantly ($p > 0.01$) related to Vvt, Svt, or total gas exchange surface area. Surface density was negatively correlated with Vvt (r_s, −0.676; $p < 0.005$) and positively correlated with total gas exchange surface area (r_s, 0.591; $p < 0.01$).

Comparisons between the Various Physiologic Variables

Static lung compliance was positively correlated with K (r_s, 0.792; $p < 0.0005$), DCO_{sb} (r_s, 0.726; $p < 0.0025$) and VAeff (r_s, 0.765; $p < 0.001$), and K was also positively correlated with VAeff (r_s, 0.711; $p < 0.0025$). The relationships between VAeff (expressed as per-

TABLE 2

MORPHOMETRIC DATA DERIVED FROM STUDY OF 16 LUNGS FROM SHEEP SEROPOSITIVE FOR MAEDI-VISNA VIRUS

Sheep No.	Symbol	Total Lung Weight (*kg*)	Total Lung Volume (*ml*)	Tissue Density (%)	Total Alveolar Volume (*ml*)	Surface Density (cm^2/cm^3)	Air-space Surface Area (m^2)
7	○	0.81	2,498	26.0	1,572	537.6	114.1
12	◐	0.72	2,188	22.0	1,451	684.9	127.4
17	⊖	0.86	3,194	23.5	2,077	542.9	147.4
32	⊕	0.55	1,750	18.0	1,451	596.4	105.5
53	⊘	1.32	2,844	31.9	1,645	410.1	99.1
59	⊖	0.84	2,716	24.0	1,754	534.1	123.3
68	⊙	1.35	2,651	43.4	1,276	374.2	84.3
73	◑	1.20	3,646	29.1	2,198	505.6	156.7
74	◐	1.74	3,055	50.2	1,294	265.4	68.9
79	◓	1.39	2,352	48.7	1,025	243.4	48.7
89	◒	0.60	2,370	17.8	1,655	578.5	116.5
90	●	0.64	2,643	19.7	1,803	676.4	152.0
92	◴	0.62	2,625	17.0	1,851	555.8	124.0
93	◶	0.60	1,823	24.9	1,164	671.4	104.0
96	+	0.96	2,534	23.5	1,648	673.1	145.0
97	•	0.61	2,425	17.5	1,700	519.7	107.1
Median		0.82	2,580	23.7	1,647	540.3	115.3
Range		0.55–1.74	1,750–3,646	17.0–50.2	1,025–2,198	243.4–684.9	48.7–156.7

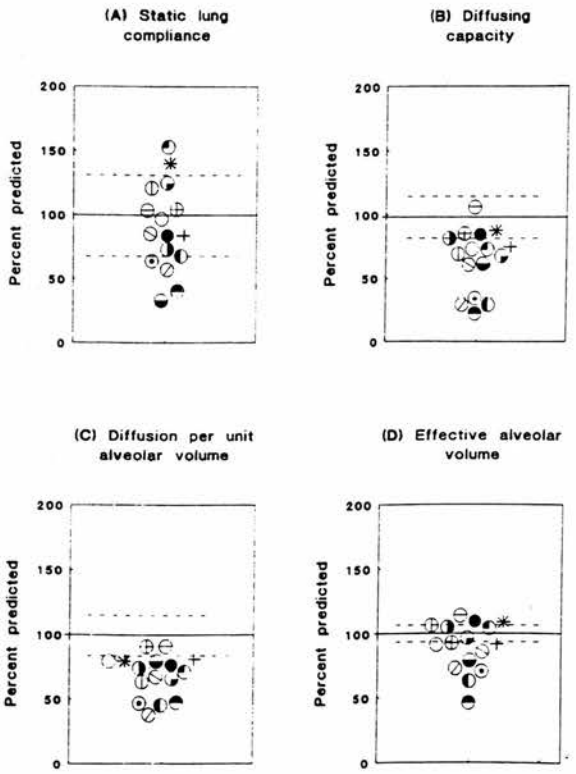

Figure 1. The relative distribution of (A) Cst, (B) DCO_{sb}, (C) D/VA, and (D) VAeff measurements from 16 sheep seropositive for MVV. Results are expressed as percent of predicted value with dotted lines representing the 95% confidence limits for normal values for sheep of the same breed and sex (14). Symbols as in Table 2.

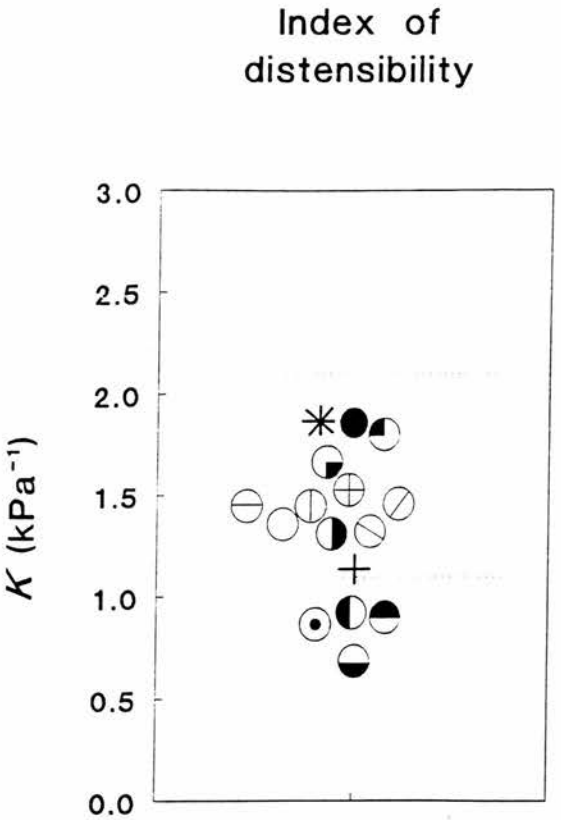

Figure 2. Distribution of K measurements for 16 sheep seropositive for MVV. The dotted lines represent the limits of the normal range for sheep of the same breed, age, and sex (Collie and coworkers, 1993; unpublished data). Symbols as in Table 2.

cent of predicted value) and Cst (r_s, 0.711; $p < 0.0025$), and between VAeff and K (r_s, 0.686; $p < 0.005$) are demonstrated in Figure 3. The significant positive correlation between DCO_{sb} and VAeff (r_s, 0.882; $p < 0.0005$) is illustrated in Figure 4.

Comparison of Morphologic with Physiologic Data

Correlation coefficients and levels of significance of the relationship between morphologic and physiologic variables are shown in Table 3. In the initial analysis, no significant correlations were demonstrated between K and the morphometric variables (Table 3); however, one K value (Sheep 89) was noted to have disproportionate influence, and this was removed (see DISCUSSION). Subsequent analysis of the reduced data set revealed highly significant correlations between K and TLW (r_s, 0.829; $p < 0.0005$), and K and Vvt (r_s, −0.764; $p < 0.001$). There was no significant correlation between K and Svt (r_s, 0.579; $0.01 < p < 0.025$). Linear regression of K (kPa^{-1}) on Svt (cm^2/cm^3) gave the equation: $K = 0.585 + (0.00154 \times Svt)$, which was significant ($R = 0.66$; $p < 0.01$) (Figure 5).

Although morphometric estimates of VAeff are significantly correlated with the physiologic measurements of the same, it is evident that there is poor agreement between these two measurements (Figure 6), with physiologic estimates considerably overestimating morphometric (median difference, 2.121; range, 0.85 to 3.191). The ratio of TVA_f to VAeff varied from 0.28 to 0.53 (median, 0.44).

DISCUSSION

In previous studies (11) we have demonstrated that sheep with MVV-induced LIP have significantly reduced lung volume and diffusing capacity values and a trend toward reduced static lung compliance values compared with those in control sheep. In this study, our intention was to select sheep demonstrating a wide spectrum of clinical and functional abnormality such that the structural basis behind these observations could be investigated. The results of this study indicate that (*1*) abnormalities of diffusion and gas exchange occur prior to significant structural abnormalities, and they are a sensitive means of assessing this disease, and (*2*) that changes in the elastic behavior of the lungs, which cannot be explained by surface-related forces, may be related to parenchymal smooth muscle hyperplasia, a pathologic feature of LIP associated with MVV infection.

The range of lung disorders represented was wide, with TLW varying from 0.55 to 1.74 kg. Based on the power law formulae of Stahl (23), the normal range of TLW for sheep of the same BW

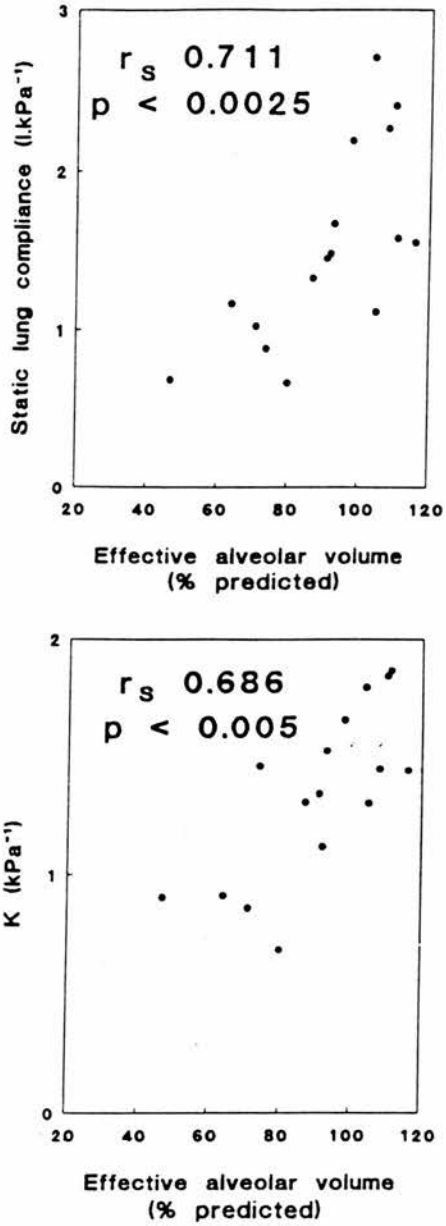

Figure 3. The relationship between Cst and VAeff, and K and VAeff, with VAeff expressed as percent of predicted value. There were significant positive correlations between these variables (r_s, 0.711; $p < 0.0025$ and r_s, 0.686; $p < 0.005$, respectively). Data from Sheep 89 are shown but not included in the analyses.

as the sheep used in this study is 0.53 to 0.73 kg. Correlations between Svt and Vvt, and body condition score and BW indicated that sheep with less structural abnormalities present in their lungs tended to be in better condition.

A larger proportion of sheep had abnormally low DCO_{sb} and

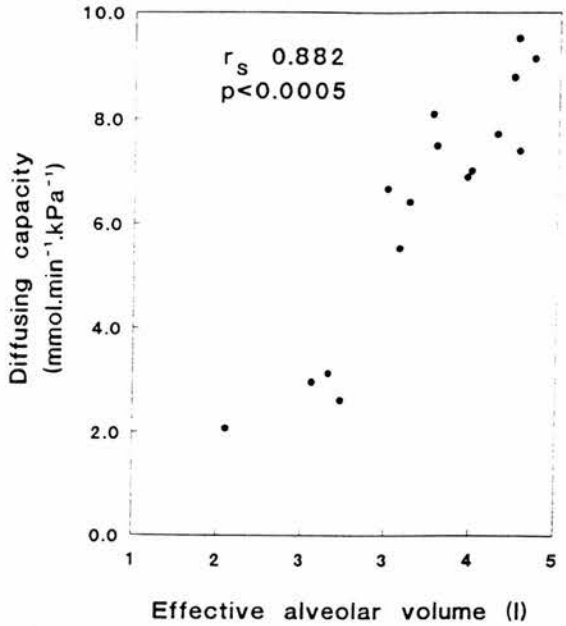

Figure 4. The relationship between VAeff and DCO_{sb}. There was a significant positive correlation (r_s, 0.882; $p < 0.0005$) between these variables.

D/VA values than low VAeff or Cst values, and reduced values of DCO_{sb} and D/VA occurred in several sheep that had little or no evidence of structural abnormality (Table 2 and Figure 1). This reflects clinical experience with human interstitial lung disease where, of the functional measurements, diffusing capacity and gas exchange studies appear to be the most sensitive indicators of alveolitis in both human idiopathic fibrosis and sarcoidosis (24). Further, it is of note that D/VA is strongly negatively correlated with TLW and Vvt, suggesting that as disease progresses, in addition to volume-related effects, factors associated with the development of the interstitial reaction such as regional inhomogeneities in the

TABLE 3

SPEARMAN'S RANK CORRELATION COEFFICIENTS (UPPER VALUES) AND LEVELS OF SIGNIFICANCE (LOWER VALUES) OF RELATIONSHIPS BETWEEN PHYSIOLOGIC AND MORPHOMETRIC VARIABLES

	Total Lung Weight	Total Lung Volume	Tissue Density	Total Alveolar Volume	Surface Density	Air-space Surface Area
Cst	−0.582	−0.324	−0.632	0.203	0.585	0.332
	< 0.01	NS	< 0.01	NS	< 0.01	NS
K	−0.555	−0.185	−0.532	0.288	0.450	0.268
	NS	NS	NS	NS	NS	NS
DCO_{sb}	−0.595	−0.088	−0.762	0.582	0.656	0.694
	< 0.01	NS	< 0.001	< 0.01	< 0.005	< 0.0025
D/VA	−0.657	−0.279	−0.721	0.426	0.576	0.524
	< 0.005	NS	< 0.0025	NS	NS	NS
VAeff	−0.515	−0.047	−0.653	0.591	0.700	0.779
	NS	NS	< 0.005	< 0.01	< 0.0025	< 0.0005

Definition of abbreviations: Cst = static compliance; K = lung distensibility; DCO_{sb} = single-breath diffusing capacity; D/VA = alveolar-volume-corrected diffusing capacity; VAeff = effective alveolar volume; NS = not significant.

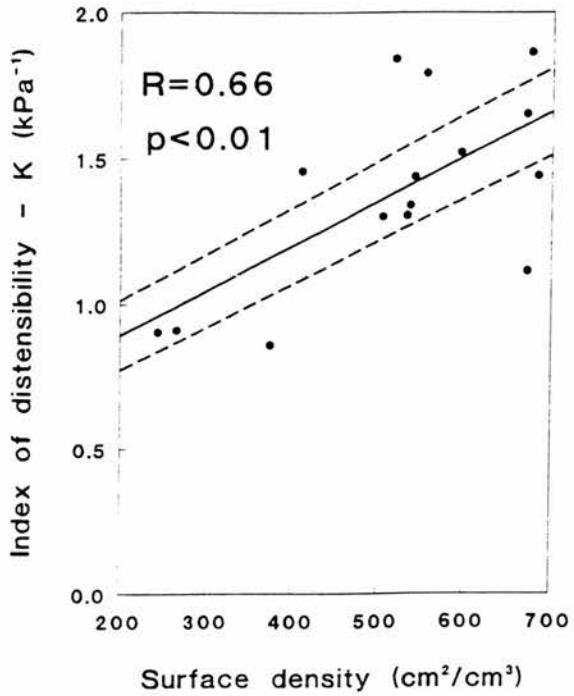

Figure 5. Regression of index of distensibility (K) on surface density (Svt). Data from Sheep 89 are not included in the analysis. The relationship is significant ($R = 0.66$; $p < 0.01$). The solid line represents the line of regression (see text), and the dotted lines depict the 95% confidence interval for the mean value of K for a given value of Svt.

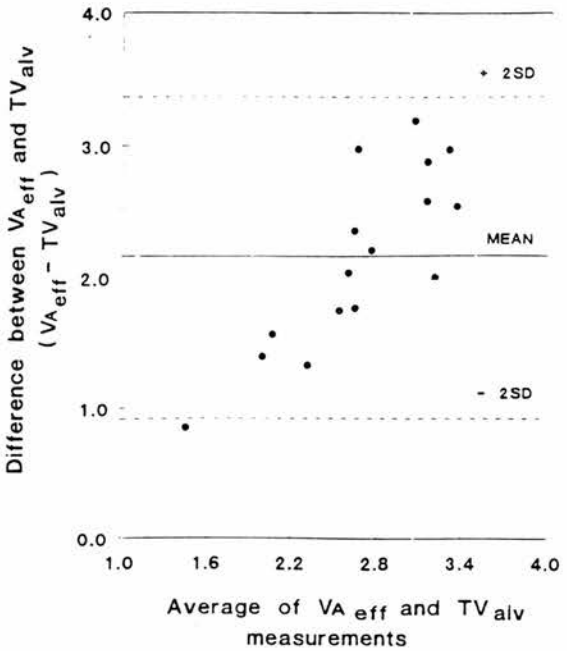

Figure 6. The agreement between morphometric and physiologic estimates of alveolar volume. There is poor agreement, with the difference between the two measurements increasing as lung volume increases.

lungs regarding blood flow, ventilation, or gas diffusing properties, reduced pulmonary capillary blood volume or thickening of the alveolar capillary membrane are important in limiting gas diffusion.

Some HIV-infected persons with no detectable lung infection or tumor have respiratory symptoms and/or abnormalities in gas exchange (25). A cytotoxic CD8 lymphocytic alveolitis is frequently identified in these subjects, and it seems to correlate with abnormal lung function indices (26). Meignan and coworkers (27) demonstrated that a reduction in DCO_{sb} and D/VA was associated with an increase in lung epithelial permeability in these patients and that the increase in permeability was highly correlated with HIV-specific cytotoxic activity. This suggested that some immunologic conflict between HIV-specific cytotoxic T-lymphocytes and HIV-infected target cells may be responsible for damage to the lung epithelium (27). In LIP associated with MVV infection there is also a CD8 T-lymphocytic alveolitis (28, 29). It is therefore possible that similar mechanisms may be responsible for the impaired gas exchange apparent in those sheep infected with MVV but showing negligible structural disorders.

Subjective and morphometric assessment suggested no pathologic change in Sheep 89. It was therefore surprising that Cst and K values were so low in this animal. This anomalous observation presumably reflected a dynamic influence peculiar to this sheep during the measurement period such as variation in the smooth muscle tone surrounding the airways, vasculature, or parenchyma or variation in pulmonary blood volume. In that this sheep did not seem representative of the structure-function relationships otherwise demonstrated, we felt justified in removing it from any analyses concerning Cst and K measurements.

There was considerable disagreement between the respective estimates of alveolar volume (VAeff and TVA) (Figure 6). An additional observation was that the difference was not consistent and tended to increase as alveolar volume estimates increased. The ratios we obtained between fixed and physiologic lung volumes are considerably lower than those obtained by Lum and Mitzner (30) for sheep lungs. Lum and Mitzner, commenting on the variability of previously reported ratios of fixed lung volume to TLC, suggested that differences were likely due to differences in fixation and/or the methodology used for air TLC determination (30). Our own observations suggest that another major source of this variation relates to the time between death and commencement of inflation fixation, i.e., with time after death, sheep lungs become progressively easier to inflate (Collie and coworkers, 1993; unpublished data). This phenomenon may be attributable to postmortem bronchonconstriction as a result of endogenous mediator release leading to gas trapping and difficulties inflating the lungs (30, 31), and it highlights the need to standardize the interval between death and inflation fixation of lungs in sheep. Given the standardized time between death and fixation in this study, it is interesting to consider which aspects of the disease process may contribute to variable tissue shrinkage.

The relative quantities of collagen and elastin fibers, i.e., "fixable and nonfixable" connective tissue (30), in the pleura and alveolar septae may influence fixed lung volume, in that after release of the inflating fixative, the elastin, which cannot be fixed (32),

may cause the lungs to recoil. It is interesting to note that Georgsson and coworkers (33) described elastic fibers to be sparse and fragmented in LIP associated with MVV infection. This fragmentation would presumably decrease recoil postfixation and lead to a relative overestimate of the fixed lung volume, in turn leading to increased fixed lung volume to TLC ratios as disease progresses, i.e., the observed relationship in this study.

An alternative to the hypothesis that tissue shrinkage is responsible for variation in the fixed to physiologic lung volume ratio is that *in vivo* lung mechanical properties are altered in disease and limit the physiologic lung volume achieved during inflation to a P_L of 3 kPa. In line with this alternative hypothesis is the observation that, in this study, there is an abnormal reduction in VAeff and that this reduction is associated with a reduction in Cst. The positive correlation between percent predicted VAeff and K indicates that the distensibility of remaining inflatable lung units is progressively reduced as lung volume is reduced, i.e., as disease progresses. Thus, diseased lungs are less distensible in LIP associated with MVV infection, and this reduction in distensibility may contribute to limitation of the lung volume achieved at a given inflating pressure, leading to an increase in the fixed lung volume to TLC ratio (Figure 6). The question then arises as to whether tissue or surface-related components are responsible for this reduced distensibility.

Haber and coworkers (34) demonstrated that, in excised mammalian lungs (rat, cat, and dog), the density of surface forces was the major determinant of lung distensibility, with distensibility being inversely related to surface density. However, it would appear that as progressive volume reduction occurs in LIP associated with MVV infection, there is a positive correlation between Svt and K (Figure 5). Therefore, the lack of distensibility in LIP associated with MVV infection is not explained by the density of surface forces. The positive correlation and significant linear relationship between K and Svt is in fact the exact opposite to perceived relationships that occur in association with advancing age (35) and with fibrosing alveolitis (36) in humans. With advancing age, an increase in the size of air spaces is associated with an increase in K (35), and in fibrosing alveolitis it is thought that there is a reduction in the size of air spaces that in turn results in a reduction in K. It therefore appears that our findings are at variance with previous investigators (34–36) who attribute variations in K to differences in the density of surface forces.

We therefore asked whether there are dynamic tissue forces present *in vivo* that are capable of influencing K but whose influence is removed during the fixation process. It is tempting to speculate as to possible causes of the observed variance in K that are only present *in vivo*.

A notable pathologic feature of LIP associated with MVV infection is a pronounced increase in contractile tissue within the pulmonary parenchyma, both at the level of the alveolar ducts and respiratory bronchioles and also extending into the interalveolar septae (33). In normal lungs, in addition to the smooth muscle in the alveolar ducts and in the walls of the pulmonary vessels and airways there is a variable amount of contractile tissue present in the pulmonary interstitium (37), which may substantially increase in amount in natural (38, 39) and in experimental fibrotic lung disease (40). The potential contribution of parenchymal contractile tissue to the overall mechanical properties of the lungs is implied by both *in vitro* (41–44) and *in vivo* (45, 46) studies. *In vivo* observations of rapid and reversible changes in lung elastic recoil in response to beta-2 agonist administration (47) and in association with exercise-induced dyspnea (48, 49) in humans provides further challenge to the traditional view that the elastic properties of the lung parenchyma are fixed, static, and virtually immutable properties that do not change rapidly with time.

Given that parenchymal smooth muscle hyperplasia is a feature of MVV-induced LIP, a potential mechanism for tissue-related forces to alter the lung elastic properties exists. Indeed it has been suggested that the increase in contractile tissue seen in MVV infection is a compensatory response for the loss of elastic recoil as a result of destruction of elastic fibers in this disease (33). That contractile tissue tone within the parenchyma might be responsible for variation in K *in vivo* is an attractive hypothesis that deserves further study.

Other potential sources of variability in K concern airway and vascular elements, in that substantial changes in lung elasticity result from contraction of the larger airways in anesthetized sheep (50), and it is known that variation in pulmonary blood volume will influence lung elasticity (51). In the latter instance any changes are likely to be minimal as even large experimental changes of pulmonary artery pressure and pulmonary blood flow leave the pressure-volume relationship of dog lungs virtually unaffected (52).

In summary, pathophysiologic correlations were examined in a group of 16 sheep seropositive for MVV infection. TLW and Vvt were increased more than twofold and Svt was reduced to half of normal in sheep with clinically obvious disease. Of the physiologic values, VAeff, DCO_{sb}, and D/VA values were less than predicted in the majority of the sheep. Strong correlations between physiologic and pathologic measurements were demonstrated. In general, measurements of DCO_{sb}, D/VA, and VAeff were more sensitive indices of disease than were measurements of Cst or K. Diffusing capacity measurements were volume-dependent, and they correlated well with volume-related parameters such as Svt, TVA, and total air-space surface area, whereas D/VA was negatively correlated with indices reflecting the extent of interstitial reaction such as TLW and Vvt. Diffusing capacity measurement values were reduced even in sheep with minimal or no morphometric evidence of disease, suggesting their value as a sensitive, if relatively nonspecific, means of assessing this condition. Tissue shrinkage differed according to the stage of the disease. The density of surface forces could not account for variation in the distensibility of the lungs; however, other factors such as the quantity and functional tone of contractile tissue in the parenchyma, airways, or blood vessels may contribute to the variation in lung distensibility seen *in vivo*.

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